



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p>(21) International Application Number: PCT/GB94/02331</p> <p>(22) International Filing Date: 21 October 1994 (21.10.94)</p> <p>(30) Priority Data: 9321861.8 22 October 1993 (22.10.93) GB 9400022.1 4 January 1994 (04.01.94) GB</p> <p>(71) Applicant (for all designated States except US): THROMBOSIS RESEARCH INSTITUTE [GB/GB]; 90 Fetter Lane, London EC4A 1EQ (GB).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): JEHANLI, Ahmed, Mohammed, Taki [GB/GB]; 1 Crescent Grove, Mitcham, Surrey CR4 4BL (GB). PATEL, Geeta [GB/GB]; 99 Bramfield Road, London SW11 6PZ (GB). OLABIRAN, Yemisi [GB/GB]; 26 Odessa Street, London SE16 1LW (GB). BRENNAND, David, Mark [GB/GB]; 30 Lawn Road, Staple Grove, Taunton TA2 6EH (GB). KAKKAR, Vijay, Vir [IN/GB]; 6 Aspen Copse, Bickley, Kent BR1 2NZ (GB).</p> <p>(74) Agents: LINN, Samuel, Jonathan et al.; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).</p>	<p>(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ).</p> <p><b>Published</b> With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</p>																																											
<p>(54) Title: PLATELET-DERIVED GROWTH FACTOR ANALOGUES</p> <table border="0"> <tr> <td><sup>25</sup>I-S-R-R-L-I-D-R-T-N-A-N-F-L<sup>38</sup></td> <td>(GP1)</td> <td></td> </tr> <tr> <td>Ac-<sup>25</sup>I-S-R-R-L-I-D-R-T-N-A-N-F-L<sup>38</sup></td> <td>(GP2)</td> <td></td> </tr> <tr> <td><sup>25</sup>I-S-R-R-L-I-D-R-T-N-A-N-F-L<sup>38</sup>-C</td> <td>(GP3)</td> <td></td> </tr> <tr> <td>Ac-<sup>25</sup>I-S-R-R-L-I-D-R-T-N-A-N-F-L<sup>38</sup>-C</td> <td>(GP4)</td> <td></td> </tr> <tr> <td><sup>25</sup>I-S-R-R-L-I-D-R-T-N-A-N-F-L-V-W-P-P-C<sup>43</sup></td> <td>(GP9)</td> <td></td> </tr> <tr> <td>Ac-<sup>25</sup>I-S-R-R-L-I-D-R-T-N-A-N-F-L-V-W-P-P-C<sup>43</sup></td> <td>(GP10)</td> <td></td> </tr> <tr> <td></td> <td></td> <td>(I)</td> </tr> <tr> <td><sup>73</sup>R-K-I-E-I-V-R-K-K<sup>81</sup></td> <td>(GP5)</td> <td></td> </tr> <tr> <td>Ac-<sup>73</sup>R-K-I-E-I-V-R-K-K<sup>81</sup></td> <td>(GP6)</td> <td></td> </tr> <tr> <td><sup>73</sup>R-K-I-E-I-V-R-K-K<sup>81</sup>-C</td> <td>(GP7)</td> <td></td> </tr> <tr> <td>Ac-<sup>73</sup>R-K-I-E-I-V-R-K-K<sup>81</sup>-C</td> <td>(GP8)</td> <td></td> </tr> <tr> <td><sup>73</sup>R-K-I-E-I-V-R-K-K-P-I-F-K-K-A-T-V<sup>89</sup></td> <td>(GP21a)</td> <td></td> </tr> <tr> <td><sup>73</sup>R-K-I-E-I-V-R-K-K-P-I-F-K-K-A-T-V<sup>89</sup>-C</td> <td>(GP21)</td> <td></td> </tr> <tr> <td>Ac-<sup>73</sup>R-K-I-E-I-V-R-K-K-P-I-F-K-K-A-T-V<sup>89</sup>-C</td> <td>(GP22)</td> <td></td> </tr> </table> <p>(57) Abstract</p> <p>Novel peptide analogues of platelet-derived growth factor, for use in inhibiting or stimulating growth and/or chemotaxis of cells, e.g. smooth muscle cells. The peptides are selected from any of (I) or linked peptides formed from pairs of the above, or such peptides including a spacer element or cyclised or modified forms thereof.</p>			<sup>25</sup> I-S-R-R-L-I-D-R-T-N-A-N-F-L <sup>38</sup>	(GP1)		Ac- <sup>25</sup> I-S-R-R-L-I-D-R-T-N-A-N-F-L <sup>38</sup>	(GP2)		<sup>25</sup> I-S-R-R-L-I-D-R-T-N-A-N-F-L <sup>38</sup> -C	(GP3)		Ac- <sup>25</sup> I-S-R-R-L-I-D-R-T-N-A-N-F-L <sup>38</sup> -C	(GP4)		<sup>25</sup> I-S-R-R-L-I-D-R-T-N-A-N-F-L-V-W-P-P-C <sup>43</sup>	(GP9)		Ac- <sup>25</sup> I-S-R-R-L-I-D-R-T-N-A-N-F-L-V-W-P-P-C <sup>43</sup>	(GP10)				(I)	<sup>73</sup> R-K-I-E-I-V-R-K-K <sup>81</sup>	(GP5)		Ac- <sup>73</sup> R-K-I-E-I-V-R-K-K <sup>81</sup>	(GP6)		<sup>73</sup> R-K-I-E-I-V-R-K-K <sup>81</sup> -C	(GP7)		Ac- <sup>73</sup> R-K-I-E-I-V-R-K-K <sup>81</sup> -C	(GP8)		<sup>73</sup> R-K-I-E-I-V-R-K-K-P-I-F-K-K-A-T-V <sup>89</sup>	(GP21a)		<sup>73</sup> R-K-I-E-I-V-R-K-K-P-I-F-K-K-A-T-V <sup>89</sup> -C	(GP21)		Ac- <sup>73</sup> R-K-I-E-I-V-R-K-K-P-I-F-K-K-A-T-V <sup>89</sup> -C	(GP22)	
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PLATELET-DERIVED GROWTH FACTOR ANALOGUES

This invention relates to platelet-derived growth factor (PDGF) analogues and their use as cell antiproliferative agents.

5        Relevant background material is incorporated herein by reference in the text to the listed references in the appended bibliography.

Platelet-derived growth factor (PDGF) is a potent mitogen for connective tissue cells and promotes the  
10 proliferation of fibroblasts and smooth muscle cells (SMC) [33]. The growth factor is a 28-31KD dimeric, highly basic ( $p_i=9.8-10$ ) glycoprotein consisting of two highly homologous (up to 60% sequence homology) polypeptide chains which are the products of distinct  
15 genes. The gene products designated A (on chromosome 7) and B (on chromosome 22) are assembled to form either a disulphide-linked heterodimer (PDGF-AB) or a homodimer (PDGF-AA or PDGF-BB). Analysis of the PDGF present in human platelets reveals that it is a mixture of all three  
20 dimeric forms with AB being the predominant form (up to 70%) [10;12]. The human prot-oncogene, c-sis, which codes for the PDGF-B chain [21] has been identified as the human homologue of the v-sis oncogene of the transforming retrovirus, simian sarcoma virus. This

oncogene codes for the protein p28 v-sis which has been identified as PDGF-BB [5].

The cloning and amino acid sequencing of the A and B chains of human PDGF have shown that both chains are synthesised as precursor molecules with hydrophobic leader sequences and both chains undergo proteolytic cleavage at the N-termini during maturation. The B chain is also processed at the C-terminal end [21;20].

The three isoforms of PDGF exert their biological effects by binding with different affinities to two different receptor types, denoted  $\alpha$  and  $\beta$ . Ligand binding induces dimerization of receptors; the A-subunit of PDGF binds to  $\alpha$ -receptors whereas the B-subunit binds to both  $\alpha$ - and  $\beta$ -receptors [2].

When PDGF dimer is treated with reducing agents, the protein loses its biological activity irreversibly, suggesting that the protein conformation is disturbed by reduction of critical disulphide bonds [16]. PDGF has 8 cysteine residues which are highly conserved between the two chains. Six residues are involved in 3 intramolecular disulphide bonds: Cys-16---Cys-60, Cys-49---Cys-97 and Cys-53---Cys-99. The other two cysteine residues are involved in asymmetrical inter-molecular disulphide bonds, Cys-43---Cys-52 [11].

A systematic analysis of the abilities of different peptides, derived from the PDGF-B chain sequence, to compete with  $^{125}\text{I}$ -PDGF-BB for binding to PDGF  $\beta$ -receptors, has led to the identification of two regions in the B-chain corresponding to amino acid residues 35-40 and 78-83 that seem important for receptor binding. A peptide corresponding to the two sequences (ANFLVW---EIVRKKP) has been found to be effective as an antagonist for PDGF, although detailed analysis has shown the pure peptide to be less active [6].

Site-directed mutagenesis studies, using deletion and substitution mutants of PDGF-BB or of the homologous v-sis gene as well as PDGF-A/B chimeras, have also identified a number of amino acid residues which are important for the biological activity of PDGF. The region Ile-25---Phe-38 has been identified as a binding domain by site directed mutagenesis of the v-sis gene [9]. Amino acid residue Asn-34 has been found to be essential for the PDGF-B-like transforming efficiency of PDGF-A/B chimera [27]. Using a different functional assay, which selects for mutants with reduced binding to both receptor types, Ile-30 and, to a lesser extent, Arg-27 have been shown to be important [3]. Basic polypeptides such as polylysine and protamine sulphate inhibit PDGF binding to its receptor, suggesting a role for ligand positive charge in the binding interaction.

A receptor binding domain has been assigned to a region at the C-terminal end which is rich with basic amino acid, residues Lys-80---Cys-97 [39]. This region contains the sequence Val-78---Arg-79---Lys-80---Lys-81-  
5 --Pro-82, which is conserved in both the A and B chains, and therefore may be involved in the binding of both chains to PDGF  $\alpha$ -receptor. A mutant PDGF-A chain in which the cationic sequence Arg-Lys-Lys has been replaced by the sequence Glu-Glu-Glu displays a marked reduction  
10 in both binding affinity for PDGF  $\alpha$ -receptor and mitogenic activity in fibroblast cells [7]. Initial studies with neutralizing monoclonal antibodies raised to PDGF-BB indicates that the segment between Thr-20 and Cys-43 represents a surface domain of PDGF-BB and  
15 contains amino acid residues involved in receptor binding [22].

Recently, the crystal structure of the homodimeric BB isoform of human recombinant PDGF has been determined [26]. The protein polypeptide chain is folded into two  
20 highly twisted anti-parallel pairs of  $\beta$ -strands and contains an unusual knotted arrangement of three intramolecular disulphide bonds. Dimerization leads to the clustering of three surface loops at each end of the elongated dimer, which most probably form the receptor  
25 recognition sites. The three loops are: loop I: Ile-25--Leu-38, loop II: Cys-53---Val-58 and loop III: Val-78---

Lys-81.

Antibodies to PDGF would be extremely useful in the study of PDGF processing and biosynthesis. It has been difficult to make high avidity antibodies against PDGF, maybe because the molecule is conserved between species and only recently have monoclonal antibodies against PDGF become available [22;34;12;38]. Rabbit and goat antisera to PDGF have been made to the two chains using protein purified from human platelets or recombinant protein or synthetic peptides, some showing chain specificity and neutralizing activity [28;17;13;37;30]. None of the antibodies raised to peptides however have been capable of recognising the native molecule or able to neutralize its biological activities.

PDGF has been implicated in many biological systems. Originally, the close similarity between PDGF and the transforming factor involved in SSV transformation led to the concept that over-production of the factor was involved in the development of human malignancies [14]. Examination of many tumour cell lines shows that the A and B chains are commonly expressed in such cell lines [15;24]. In general, aberrant expression of PDGF or of PDGF receptors is likely to be involved in the stimulation of the growth of certain tumours. In addition, over-activity of PDGF could also be part of the

development of certain non-malignant disorders involving an excess of cell proliferation. Examples include atherosclerosis, where PDGF-induced stimulation of smooth muscle cell proliferation could contribute to the thickening of the intima of affected vessels [32], as well as chronic fibrotic processes, where PDGF could be involved in the stimulation of connective tissue cell proliferation. Ferns et al [8] showed that in a rat experimental model of angioplasty, polyclonal antibodies to PDGF administered intravenously inhibited smooth muscle cell accumulation in the intima of injured arteries, while administration of PDGF induced SMC proliferation in the media by 2-3 fold and, more significantly, increased SMC migration from the media to the intima by 20-fold [19].

However, PDGF does have a normal function. PDGF and PDGF receptors are expressed in embryonic tissues and in the placenta [23;18] which suggests a function for PDGF during development. A role for PDGF in neuronal development has also been proven [25] and PDGF and its receptors are present in the peripheral and central nervous systems [40;36]. PDGF is known to stimulate growth as well as chemotaxis of connective tissue cells and also chemotaxis of inflammatory cells, which suggests a role in wound healing [4;35]. Recently, PDGF has been used in a clinical trial to look at its wound healing

capability. Locally applied PDGF stimulates the healing of large bed sores [31]. PDGF  $\beta$ -receptors occur on capillary endothelial cells [29] and PDGF has weak angiogenic activity [29] which may suggest that its  
5 stimulatory effect is important in wound healing.

The varied roles of PDGF, both beneficial and adverse, make PDGF agonists and antagonists highly desirable. They can be used as a replacement for PDGF in wound healing or as inhibitors of the adverse effects of  
10 PDGF. Antibodies with neutralizing activity, whether to the mitogenic effect of PDGF and/or the chemotactic effect can also be useful as inhibitors of PDGF adverse effects.

Accordingly, in one aspect the present invention  
15 provides novel PDGF peptide analogues and compositions consisting of or containing them for use as antiproliferative agents, particularly antiatherosclerotic, antiatherogenetic, anti-inflammatory or antifibrotic agents. The invention also provides such  
20 novel PDGF peptide analogues and compositions consisting of or containing them for use as PDGF agonists for use in wound healing.

Particular PDGF analogues according to the present invention are identified in Table 1 hereinbelow.

Preferably, the PDGF peptide analogues of the invention, as prepared and used in other aspects and embodiments of the invention, are greater than about 90% pure, more preferably greater than about 95% pure, even more  
5 preferably greater than about 99% pure.

Pharmaceutical compositions in accordance with the present invention preferably comprise one or more of the PDGF analogues of the invention together with a pharmaceutically acceptable diluent and/or carrier.  
10 Suitable carriers/diluents are well known in the art and include saline or other sterile aqueous media, optionally including additional components such as buffer salts and preservatives, or sugars, starches, salts or mixtures thereof.

15 Peptides according to the present invention may be provided for use in any suitable form appropriate to the protocol of administration and/or the needs of a patient.

Apart from the pharmaceutically acceptable compositions referred to above, the peptides may for  
20 example be provided, either singly or in combination, in lyophilized or freeze dried solid forms.

Within the scope of the invention are linked peptides comprising a first analogue selected from the

group consisting of GP1, GP2, GP3, GP4, GP9 and GP10 (as identified in Table 1 hereinbelow) and a second peptide analogue selected from the group consisting of GP5, GP6, GP7, GP8, GP21a, GP21 and GP22 (as identified in Table 1  
5 hereinbelow),

The invention further provides the novel PDGF peptide analogues for use in assays and kits for assays.

It is to be understood that within the scope of the present invention are peptide analogues as described and  
10 identified herein in which one or more amino acids are substituted with other amino acids, or in which there is inserted a spacer, for example a dithiol group or a diamino group or multiples of amino acid residues, e.g. glycine, as shown in Table 2 hereinbelow, peptides GP11,  
15 GP12, GP13 and GP14. The spacer may also be a homo- or hetero-bifunctional crosslinker, for example the heterobifunctional crosslinker N-(4-carboxy-cyclohexyl-methyl)-maleimide, as shown in Table 3 hereinbelow, peptides GP20 and GP23, providing generally of course  
20 that the essential activity of the peptide remains substantially unchanged.

The invention further provides the synthesis and use of cyclic peptides such as those derived from GP4 and GP8 as shown in Table 4 below, peptides GP24 and GP25.

The invention further provides the novel PDGF peptide analogues for use in assays and kits for assays, either in the free form or linked to a carrier molecule such as a protein or a solid particle, as well as  
5 modified peptides with e.g. biotin or fluorescein isothiocyanate, such as those shown in Table 5 hereinbelow, peptides GP15, GP16, GP19, GP17 and GP18.

In a second aspect, the present invention provides a method of inhibiting or stimulating cell proliferation,  
10 particularly smooth muscle cell, 3T3-fibroblast cell, connective tissue cell or inflammatory cell proliferation, by use or administration, particularly to a host, of an effective amount of a PDGF peptide analogue as defined above.

15 The invention further provides a method of inhibiting or stimulating PDGF-induced DNA synthesis comprising use or administration, such as to a host, of an effective amount of a PDGF peptide analogue as defined above.

20 In a further aspect, the present invention provides PDGF peptide analogues as defined above for use in inhibiting or stimulating growth and/or chemotaxis of cells such as those identified above.

In yet a further aspect, the present invention provides the above-defined PDGF peptide analogues, particularly the linked peptide analogues of the invention, for use as immunogens for the production of polyclonal and monoclonal antibodies to PDGF, especially for diagnostic, prognostic and therapeutic uses. Such methods of production of polyclonal and monoclonal antibodies are also within the scope of the invention.

In yet another aspect of the present invention, the novel PDGF analogues are provided for and used in methods of inhibiting PDGF-induced DNA synthesis, for example by use of or administration of an effective amount of one or more of the above defined PDGF peptide analogues.

Administration of peptides of the invention in any of the methods described herein may be via any suitable protocol. Preferably, administration to a host, especially a human host, is by intravenous injection or infusion, and may be systemic or topical. Such administration of peptides of the invention is in such an amount as to give the desired effective result of the peptide's activity at the intended site. Thus, a quantity which constitutes an "effective" amount may depend upon various parameters, such as body weight of the patient, degree of activity required, intended site of activity, severity of the condition to be treated or

prevented, all of which will be well understood and appreciated by persons skilled in the art.

Generally, an amount (or total amount) of peptide will be administered which gives a concentration in plasma of from about 1 to about 100 mg ml<sup>-1</sup>, more preferably from about 1 to about 10 mg ml<sup>-1</sup>.

The present invention will now be described in further detail, with reference to the accompanying drawings, in which:-

10        Figure 1 shows relative mitogenic effects of various PDGF related peptides;

         Figures 2A and 2B show the results of a <sup>125</sup>I-PDGF binding assay, as described further below;

         Figures 3A and 3B show the results of titrations of,  
15        respectively, anti-Tg-GP4 vs.GP4 and anti-Tg-GP8 vs.GP8;

         Figures 4A and 4B show the results of titrations of, respectively, anti-Tg-GP4 vs.PDGF-BB and anti-Tg-GP4 vs.FGF and EGF;

         Figures 5A and 5B show the results of titrations of  
20        selected poly- and monoclonal antibodies by direct ELISA against PDGF-BB;

         Figure 6 shows the inhibition of radiolabelled PDGF-BB binding to human smooth muscle cells by anti-peptide antibodies; and

Figures 7A and 7B, 8A and 8B, and 9A and 9B show the HPLC and mass spectroscopy profiles of peptides GP4, GP8 and GP14, respectively.

#### METHODS

##### 5 1) Synthesis of PDGF-BB Peptide Analogues

A series of PDGF-BB related peptides were synthesised, with or without modifications, by solid phase on a Milligen 9050 Pepsynthesizer, using the FMOC - polyamide continuous method, as listed in Table 1  
10 hereinbelow.

Acetylation of the N-terminal end of the peptides was performed after the completion of the synthesis. The resin was acetylated on the solid-support with 45% acetic anhydride in dimethylformamide. Deprotection and  
15 cleavage of the resin were carried out in the normal manner.

Biotinylation and FITC labelling were carried out while the peptides were still attached to the resin and prior to deprotection. Biotin-caproate-N-  
20 hydroxysuccinimide (B-NHS) and fluorescein isothiocyanate were used to label the free N-terminal end of the peptides.

All peptides were purified to at least 95% homogeneity by HPLC and their molecular weights determined by mass spectroscopy. Figures 7, 8 and 9 show examples of the HPLC and mass spectroscopy profiles of 5 peptides GP4, GP8 and GP14, respectively.

2) Effect of PDGF Peptides on Fibroblast Cells in Culture

The stimulatory or inhibitory effect of the peptides on the murine fibroblast cell line Swiss 3T3.A31 were 10 investigated using the [ $^3\text{H}$ ]-thymidine uptake assay as described by Raines & Ross [28].

3) Effect of PDGF Peptides on  $^{125}\text{I}$ -PDGF-BB Binding to 3T3 Cells and Human Smooth Muscle Cells

PDGF-BB binding inhibition assay was performed as 15 described by Engstrom et al [6]. A murine fibroblast cell line 3T3.A31 and human aortic smooth muscle cells were used.

4) Production of Antisera to PDGF-Peptides

Rabbits and mice were immunised with the peptides 20 either in the free form mixed with Freund's adjuvant or

conjugated to a carrier protein (Thyroglobulin or keyhole haemocyanin). Antisera were tested for antibody production to the peptides and PDGF using ELISA, dot blot assays and SDS-PAGE followed by Western blotting.

5    5)    Effect of Anti-PDGF peptides antibodies on <sup>125</sup>I-PDGF binding to Human Smooth Muscle cells

The IgGs of the polyclonal anti- PDGF peptides antisera were purified from the antisera by affinity chromatography on a protein G -Sepharose column as  
10 described by the manufacturers (Pharmacia, Uppsala, Sweden). The effect of the IgG on the binding of radiolabelled PDGF-BB to human smooth muscle cells was investigated using essentially the same procedure as for the peptides (method 3 above). In the test, peptides  
15 were replaced with IgG.

Results

The peptides were tested for their ability to stimulate thymidine uptake in the cells in culture.

Figure 1 shows an example of the results obtained  
20 with some of the peptides. Peptide GP4 showed the highest stimulatory effect acting as an agonist for PDGF-BB. The mitogenic effect of GP4 was almost completely

abolished upon reduction and alkylation of the C-terminal end cysteine residue. This strongly suggests that the peptide is acting via the formation of a dimeric form during the incubation with the cells and that it is the  
5 dimerisation which produces the increase in the stimulatory activity. This conclusion is also supported by the low stimulatory effect of peptide GP2 which has the same amino acid sequence as GP4 but without the C-terminal cysteine.

10 Peptide GP8 was not as stimulatory as GP4.

Some of the peptides were tested for their ability to inhibit the binding of radiolabelled PDGF-BB to 3T3 cells. Both GP4 and GP8 showed modest inhibition of binding at the concentrations tested, as illustrated in  
15 Figure 2A. Peptides GP20 and GP14 were potent inhibitors of labelled PDGF binding to human smooth muscle cells, as shown in Figure 2B.

Rabbits immunised with GP4 and GP8 peptides linked to thyroglobulin produced high titre antibodies to the  
20 corresponding immunising peptide as determined by ELISA, as illustrated in Figures 3A and 3B.

One of the rabbits immunised with GP4 also produced antibodies reactive with native PDGF-BB, and had no cross

reactivity with human recombinant fibroblast growth factor (FGF) and epidermal growth factor (EGF). This is illustrated in Figure 4.

Tables 6, 7 and 8 hereinbelow summarise the results of immunochemical characterisation of polyclonal and monoclonal antisera raised to PDGF - derived peptides.

Western immunoblot analysis of polyclonal antisera reactivity with native and reduced PDGF-BB (Table 6) shows that peptides GP4 and GP21a produced antibodies that reacted with the native PDGF. The competitive ELISA data are shown in Table 7. 15 monoclonal antibody hybridomas raised to peptide GP4 coupled to thyroglobulin were immunochemically characterised as shown in Table 8. Figures 5A and 5B show typical titration curves for polyclonal and monoclonal antisera against PDGF-BB.

The IgG fraction from rabbits immunised with peptides GP4 and GP21a were effective in inhibiting the binding of radio-labelled PDGF-BB to human smooth muscle cells in culture, as shown in Figure 6.

Table 1PDGF-B CHAIN PEPTIDESLOOP I

<sup>25</sup> I-S-R-R-L-I-D-R-T-N-A-N-F-L <sup>38</sup>	GP1
Ac- <sup>25</sup> I-S-R-R-L-I-D-R-T-N-A-N-F-L <sup>38</sup>	GP2
<sup>25</sup> I-S-R-R-L-I-D-R-T-N-A-N-F-L <sup>38</sup> -C	GP3
Ac- <sup>25</sup> I-S-R-R-L-I-D-R-T-N-A-N-F-L <sup>38</sup> -C	GP4
<sup>25</sup> I-S-R-R-L-I-D-R-T-N-A-N-F-L-V-W-P-P-C <sup>43</sup>	GP9
Ac- <sup>25</sup> I-S-R-R-L-I-D-R-T-N-A-N-F-L-V-W-P-P-C <sup>43</sup>	GP10

LOOP III

<sup>73</sup> R-K-I-E-I-V-R-K-K <sup>81</sup>	GP5
Ac- <sup>73</sup> R-K-I-E-I-V-R-K-K <sup>81</sup>	GP6
<sup>73</sup> R-K-I-E-I-V-R-K-K <sup>81</sup> -C	GP7
Ac- <sup>73</sup> R-K-I-E-I-V-R-K-K <sup>81</sup> -C	GP8
<sup>73</sup> R-K-I-E-I-V-R-K-K-P-I-F-K-K-A-T-V <sup>89</sup>	GP21a
<sup>73</sup> R-K-I-E-I-V-R-K-K-P-I-F-K-K-A-T-V <sup>89</sup> -C	GP21
Ac- <sup>73</sup> R-K-I-E-I-V-R-K-K-P-I-F-K-K-A-T-V <sup>89</sup> -C	GP22

Table 2

**PDGF-B CHAIN PEPTIDES**  
**(LOOP I & LOOP III using Glycyl spacers)**

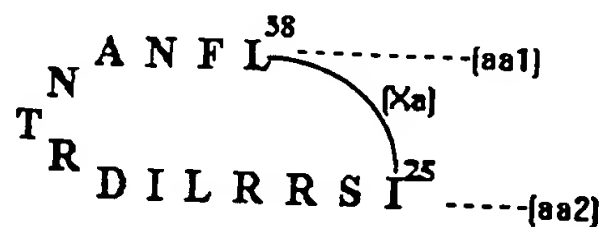
$^{25}\text{I-S-R-R-L-I-D-R-T-N-A-N-F-L}^{38}\text{-(G-G-G-G)-}$ $^{73}\text{R-K-I-E-I-V-R-K-K}^{81}\text{-C}$	GP11
$\text{Ac-}^{25}\text{I-S-R-R-L-I-D-R-T-N-A-N-F-L}^{38}\text{-(G-G-G-G)-}$ $^{73}\text{R-K-I-E-I-V-R-K-K}^{81}\text{-C}$	GP12
$^{25}\text{I-S-R-R-L-I-D-R-T-N-A-N-F-L}^{38}\text{-(G-G-G-G-G-G)-}$ $^{73}\text{R-K-I-E-I-V-R-K-K}^{81}\text{-C}$	GP13
$\text{Ac-}^{25}\text{I-S-R-R-L-I-D-R-T-N-A-N-F-L}^{38}\text{-(G-G-G-G-G-G)-}$ $^{73}\text{R-K-I-E-I-V-R-K-K}^{81}\text{-C}$	GP14

Table 3CROSS-LINKED PDGF LOOP I & LOOP III PEPTIDES

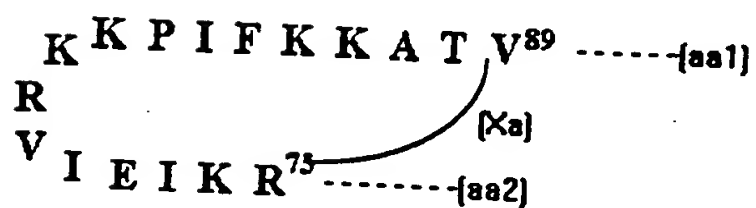
Ac-<sup>25</sup>I-S-R-R-L-I-D-R-T-N-A-N-F-L-V-W-P-P-C<sup>43</sup>-(*SMCC*)-  
<sup>73</sup>R-K-I-E-I-V-R-K-K<sup>81</sup>-C GP20

Ac-<sup>25</sup>I-S-R-R-L-I-D-R-T-N-A-N-F-L<sup>38</sup>-C-(*SMCC*)-  
<sup>73</sup>R-K-I-E-I-V-R-K-K<sup>81</sup>-C GP23

{*SMCC* :- N-(4-carboxy-cyclohexyl-methyl)-maleimide OR any  
heterobifunctional cross-linker}

Table 4CYCLIC PDGF-B CHAIN PEPTIDESLOOP I

GP24

LOOP III

GP25

(Xa = bridging spacer arm)  
 aa1 = amino acid / acids of C-terminus  
 aa2 = amino acid / acids of N-terminus

Table 5AFFINITY-LABELLED PDGF-B CHAIN PEPTIDESLOOP I

X- <sup>25</sup> I-S-R-R-L-I-D-R-T-N-A-N-F-L <sup>38</sup>	GP15
X- <sup>25</sup> I-S-R-R-L-I-D-R-T-N-A-N-F-L <sup>38</sup> -C	GP16
X- <sup>25</sup> I-S-R-R-L-I-D-R-T-N-A-N-F-L-V-W-P-P-C <sup>43</sup>	GP19

LOOP III

X- <sup>73</sup> R-K-I-E-I-V-R-K-K <sup>81</sup>	GP17
X- <sup>73</sup> R-K-I-E-I-V-R-K-K <sup>81</sup> -C	GP18

(X = Biotin or FITC)

**Table 6**  
**Polyclonal anti-PDGF peptides antisera analysis by western Blot.**

Antibody	Immunogen	vs PDGF Ser-1/100	vs PDGF Ser-1/1000	vs PDGF Ser-1/10000	vs RED-PDGF Ser-1/100	vs RED-PDGF Ser-1/10000
Rb 86	GP4	-	-	-	++	-
Rb 65	Tg-GP4	++++	++	-	++++	++++
Rb 66	Tg-GP4	++	++	-	++++	++
Rb 109	GP10	-	-	-	++	-
Rb 37	GP10	-	-	-	++	+
Rb 38	Tg-GP10	-	-	-	++	++
Rb 39	Tg-GP10	-	-	-	++++	++++
Rb 112	Tg-GP10	-	-	-	++	+
Rb 67	Tg-GP8	-	-	-	++++	++
Rb 68	Tg-GP8	+	-	-	++	-
Rb 78	GP21a	++++	++++	+	++++	++++
Rb 91	GP21a	-	-	-	++ (1/200)	++ (1/20,000)
Rb113	Tg-GP4	- (1/200)	-	-	- (1/200)	- (1/20,000)
Rb114	Tg-GP4	++ (1/200)	++ Weak + Very weak - Negative	-	++ (1/200)	++ (1/20,000)

**Table 7**  
**Competitive ELISA analysis of polyclonal anti-PDGF-BB peptides antisera**

Antibody	Immunogen	titre	GP4 IC50	GP10 IC50	GP21a IC50	GP8 IC50	PDGF IC50
Rb 86	GP4						
Rb 65	Tg-GP4	1/243,000	3nM	3nM	NONE	> 6000nM	180nM
Rb 66	Tg-GP4	1/27,000	<2nM	<2nM	NONE	NONE	NONE
Rb 109	GP10	1/10,000	2nM	4nM	NONE	NONE	NONE
Rb 37	GP10	1/27,000	20nM	10nM	NONE	NONE	NONE
Rb 38	Tg-GP10	1/27,000	150nM	74nM	NONE	NONE	NONE
Rb 39	Tg-GP10	1/100,000	2nM	2nM	NONE	NONE	NONE
Rb 112	Tg-GP10	1/243,000	2nM	3nM	NONE	NONE	NONE
Rb 67	Tg-GP8	1/243,000	Not Sig	Not Sig	Not Sig	2nM	200nM
Rb 68	Tg-GP8	1/243,000	Not Sig	Not Sig	Not Sig	<2nM	30nM
Rb 78	GP21a	1/15,000	NONE	NONE	100nM	NONE	NONE
Rb 91	GP21a	ND	ND	ND	ND	ND	ND
Rb 113	Tg-GP4	1/100,000					
Rb 114	Tg-GP4	1/2,000					

24

Peptides tried up to 6000nM, PDGF up to 200nM

**Table 8**

Reactivities of monoclonal antibodies to peptide GP4  
sub-class, ELISA, CELIA and Western blot analysis

ANTIBODY	Sub-class	ELISA TITRE	ELISA PDGF *	BLOT RED PDGF	BLOT PDGF	CELIA GP4	CELIA GP10	CELIA GP21a	CELIA GP8	CELIA PDGF **
1DMB	IgG1	ND	-ve	+	-	-	-	-	-	-
2DMB	IgG1	ND	-ve	+	-	2uM	2uM	-	-	-
3DMB	IgG1	ND	-ve	+	-	-	-	-	-	-
4DMB	IgG1	ND	-ve	+++	-	150nM	150nM	-	-	-
9DB-1	IgG1	1/10	-ve	-	-	1uM	1.2uM	-	-	+10%?
10DB-1	IgG1	1/243	10%	+++++	-	400nM	>6uM	-	-	+10%?
11DB-1	IgM	1/2	30%	++	-	-	-	-	-	+175%?
12DB-1	IgG1	1/243	-ve	+++++	-	2uM	-	-	-	25
13DB-1	IgG1	1/10	-ve	-	-	200nM	400nM	-	-	-
15DB-1	IgM	1/9	31%	++	-	-	-	-	-	+356%?
17DB-1	IgG1	1/81	30%	+++++	-	180nM	3uM	-	-	+25%?
19DB-1	IgG1	1/1000	-ve	-	-	18nM	18nM	-	-	+10%?
21DB-1	IgG1	1/1000	-ve	-	-	18nM	30nM	-	-	-
22DB-1	IgG1	1/1000	-ve	-	-	20nM	25nM	-	-	-

\* Expressed as a percentage of OD given by 500ng/ml Rb anti-PDGF (Bachem)

\*\* An increase in signal may be caused by cross-linking

In CELIAS, peptides tried up to 8000nM, PDGF up to 200nM

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CLAIMS:

1. A platelet-derived growth factor peptide analogue selected from any of the following:

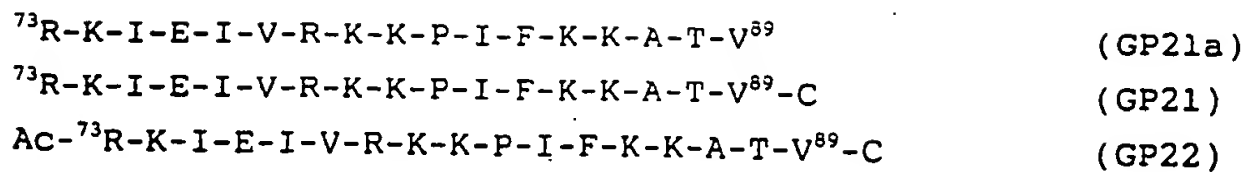
<sup>25</sup> I-S-R-R-L-I-D-R-T-N-A-N-F-L <sup>38</sup>	(GP1)
Ac- <sup>25</sup> I-S-R-R-L-I-D-R-T-N-A-N-F-L <sup>38</sup>	(GP2)
<sup>25</sup> I-S-R-R-L-I-D-R-T-N-A-N-F-L <sup>38</sup> -C	(GP3)
Ac- <sup>25</sup> I-S-R-R-L-I-D-R-T-N-A-N-F-L <sup>38</sup> -C	(GP4)
<sup>25</sup> I-S-R-R-L-I-D-R-T-N-A-N-F-L-V-W-P-P-C <sup>43</sup>	(GP9)
Ac- <sup>25</sup> I-S-R-R-L-I-D-R-T-N-A-N-F-L-V-W-P-P-C <sup>43</sup>	(GP10)
<sup>73</sup> R-K-I-E-I-V-R-K-K <sup>81</sup>	(GP5)
Ac- <sup>73</sup> R-K-I-E-I-V-R-K-K <sup>81</sup>	(GP6)
<sup>73</sup> R-K-I-E-I-V-R-K-K <sup>81</sup> -C	(GP7)
Ac- <sup>73</sup> R-K-I-E-I-V-R-K-K <sup>81</sup> -C	(GP8)
<sup>73</sup> R-K-I-E-I-V-R-K-K-P-I-F-K-K-A-T-V <sup>89</sup>	(GP21a)
<sup>73</sup> R-K-I-E-I-V-R-K-K-P-I-F-K-K-A-T-V <sup>89</sup> -C	(GP21)
Ac- <sup>73</sup> R-K-I-E-I-V-R-K-K-P-I-F-K-K-A-T-V <sup>89</sup> -C	(GP22)

2. A platelet-derived growth factor peptide analogue comprising a first sequence selected from any of the following:

<sup>25</sup> I-S-R-R-L-I-D-R-T-N-A-N-F-L <sup>38</sup>	(GP1)
Ac- <sup>25</sup> I-S-R-R-L-I-D-R-T-N-A-N-F-L <sup>38</sup>	(GP2)
<sup>25</sup> I-S-R-R-L-I-D-R-T-N-A-N-F-L <sup>38</sup> -C	(GP3)
Ac- <sup>25</sup> I-S-R-R-L-I-D-R-T-N-A-N-F-L <sup>38</sup> -C	(GP4)
<sup>25</sup> I-S-R-R-L-I-D-R-T-N-A-N-F-L-V-W-P-P-C <sup>43</sup>	(GP9)
Ac- <sup>25</sup> I-S-R-R-L-I-D-R-T-N-A-N-F-L-V-W-P-P-C <sup>43</sup>	(GP10)

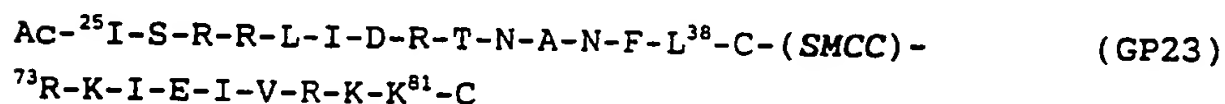
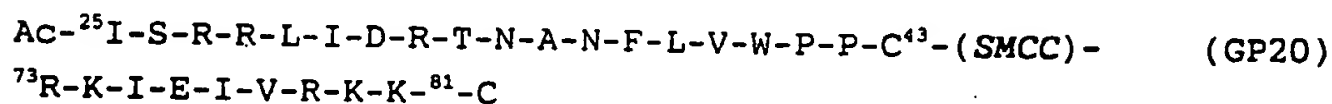
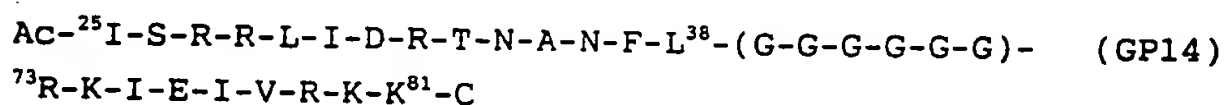
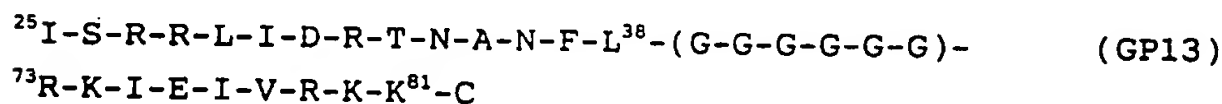
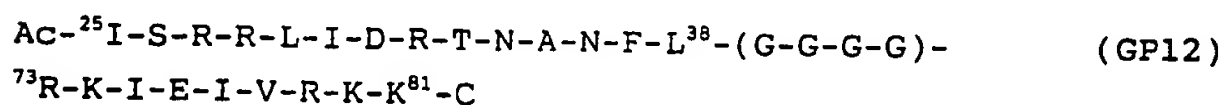
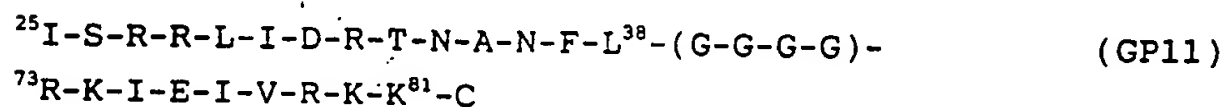
and a second sequence linked to the first sequence and selected from any of the following:

<sup>73</sup> R-K-I-E-I-V-R-K-K <sup>81</sup>	(GP5)
Ac- <sup>73</sup> R-K-I-E-I-V-R-K-K <sup>81</sup>	(GP6)
<sup>73</sup> R-K-I-E-I-V-R-K-K <sup>81</sup> -C	(GP7)
Ac- <sup>73</sup> R-K-I-E-I-V-R-K-K <sup>81</sup> -C	(GP8)



3. A peptide according to claim 1 or claim 2, wherein the amino acid sequence contains a spacer element selected from a dithiol group, a diamino group, multiples of an amino acid residue, or a homo- or hetero- bifunctional crosslinker.

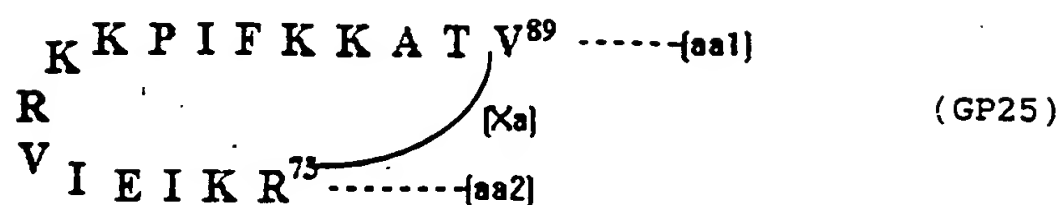
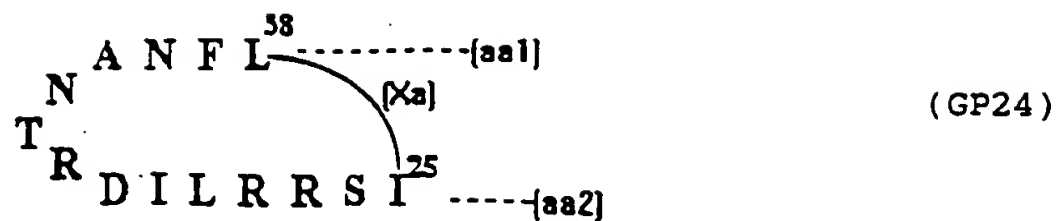
4. A peptide according to claim 3, which is selected from any of the following:



wherein SMCC is N-(4-carboxy-cyclohexyl-methyl)-maleimide or any other heterobifunctional cross-linker.

5. A peptide according to claim 1 or claim 2, which is in cyclised form.

6. A peptide according to claim 5, which is selected from any of the following:



wherein:

Xa = bridging spacer arm

aa1 = amino acid/acids of C-terminus

aa2 = amino acid/acids of N-terminus.

7. A peptide according to claim 1 or claim 2, which is linked to a carrier molecule selected from a protein or a solid particle.

8. A peptide according to claim 1 or claim 2, which is a modified peptide selected from any of the following:

X-<sup>25</sup>I-S-R-R-L-I-D-R-T-N-A-N-F-L<sup>38</sup> (GP15)

X-<sup>25</sup>I-S-R-R-L-I-D-R-T-N-A-N-F-L<sup>38</sup>-C (GP16)

X-<sup>25</sup>I-S-R-R-L-I-D-R-T-N-A-N-F-L-V-W-P-P-C<sup>43</sup> (GP19)

X-<sup>73</sup>R-K-I-E-I-V-R-K-K<sup>81</sup> (GP17)

X-<sup>73</sup>R-K-I-E-I-V-R-K-K<sup>81</sup>-C (GP18)

wherein X = Biotin or FITC..

9. A peptide according to any one of claims 1 to 8, which has

a purity greater than 90%, preferably greater than 95%.

10. A peptide according to any preceding claim for pharmaceutical use.
11. A peptide according to claim 10 for use in inhibiting or stimulating growth and/or chemotaxis of cells.
12. A peptide according to claim 11 for use in inhibiting or stimulating growth and/or chemotaxis of smooth muscle cells, 3T3-fibroblast cells, connective tissue cells or inflammatory cells.
13. A peptide analogue according to any one of claims 1 to 8 for use as an immunogen for the production of polyclonal and monoclonal antibodies to platelet-derived growth factor.
14. A peptide according to any one of claims 1 to 8, for use in inhibiting or stimulating platelet-derived growth factor-induced DNA synthesis.
15. Use of a peptide according to any one of claims 1 to 8 as a cell antiproliferative agent.
16. Use according to claim 15, wherein the cells are smooth muscle cells, 3T3-fibroblast cells, connective tissue cells or inflammatory cells.
17. Use of a peptide according to any one of claims 1 to 8 for wound healing.
18. Use of a peptide according to any one of claims 1 to 8 for inhibiting or stimulating platelet-derived growth factor-induced DNA synthesis.
19. Use of a peptide according to any one of claims 1 to 8 as an immunogen in the production of polyclonal and monoclonal antibodies to platelet-derived growth factor.
20. A method of inhibiting or stimulating growth and/or

chemotaxis of cells comprising administering to a host an effective amount of a peptide according to any one of claims 1 to 8.

21. A method according to claim 20, wherein the cells are selected from smooth muscle cells, 3T3-fibroblast cells, connective tissue cells or inflammatory cells.

22. A method of inhibiting or stimulating platelet-derived growth factor-induced DNA synthesis, comprising administering to a host an effective amount of a peptide according to any one of claims 1 to 8.

23. A method of promoting wound healing comprising administering thereto or to a host an effective amount of a peptide according to any one of claims 1 to 8.

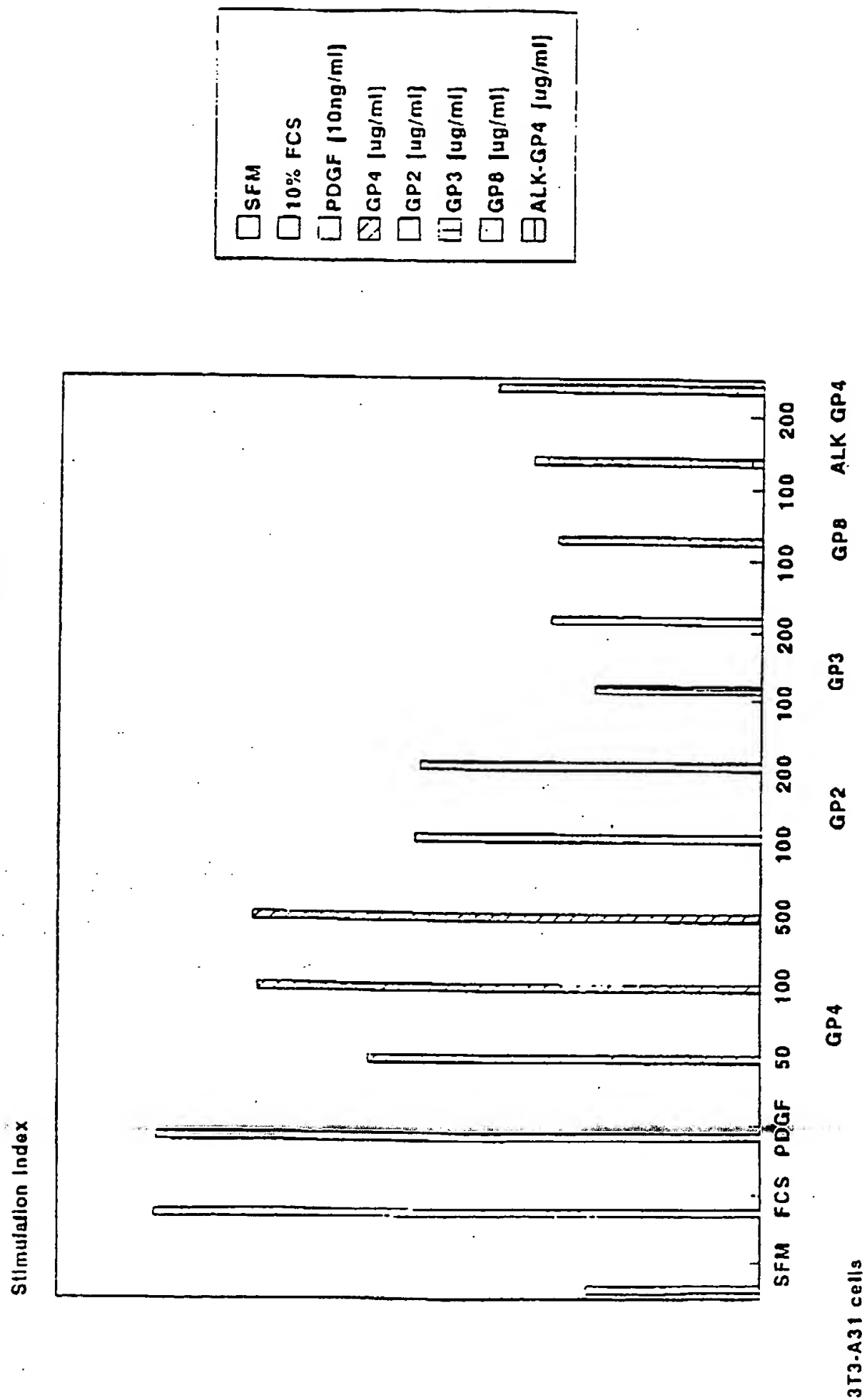
24. A pharmaceutical composition comprising one or more peptides according to any one of claims 1 to 8, together with a pharmaceutically acceptable diluent and/or carrier.

25. A pharmaceutical composition according to claim 24, wherein the peptide(s) is present in an amount such as to give a concentration thereof in plasma of a host to which the composition is administered of from 1 to 100 mg ml<sup>-1</sup>.

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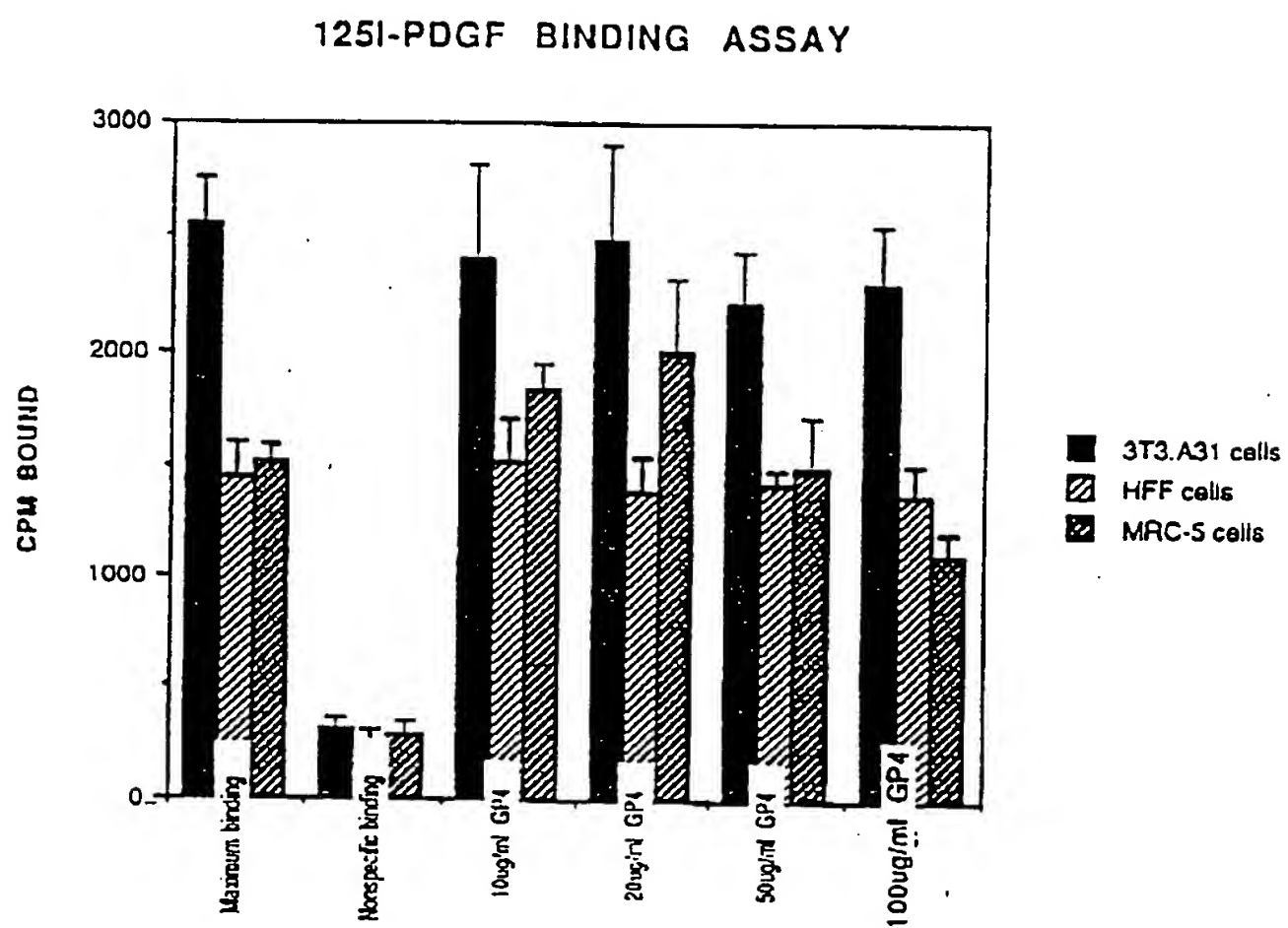
# **Mitogenic Effect of PDGF Related Peptides** Thymidine Uptake Assay

**Figure 1**



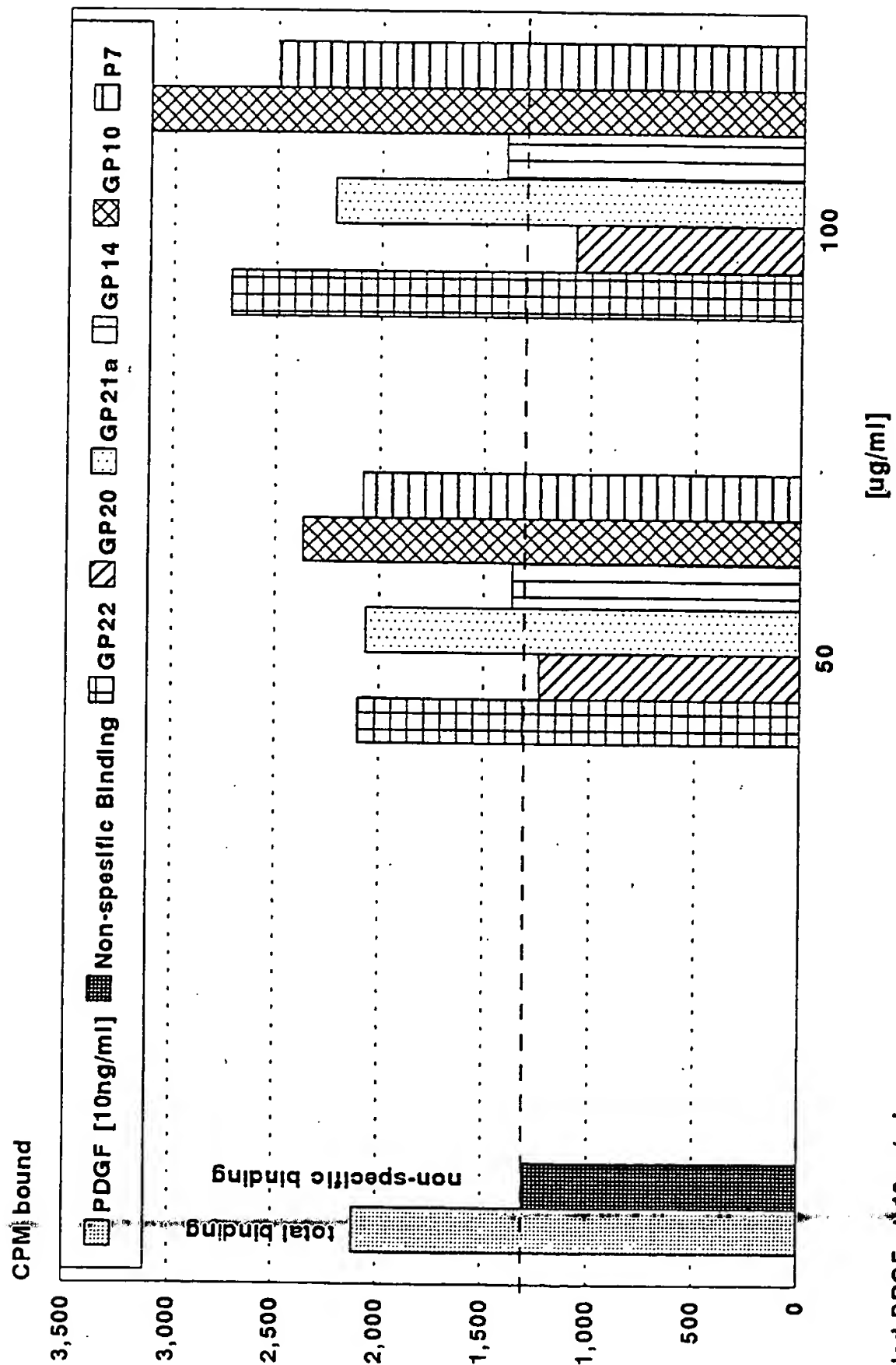
2/16

Figure 2A



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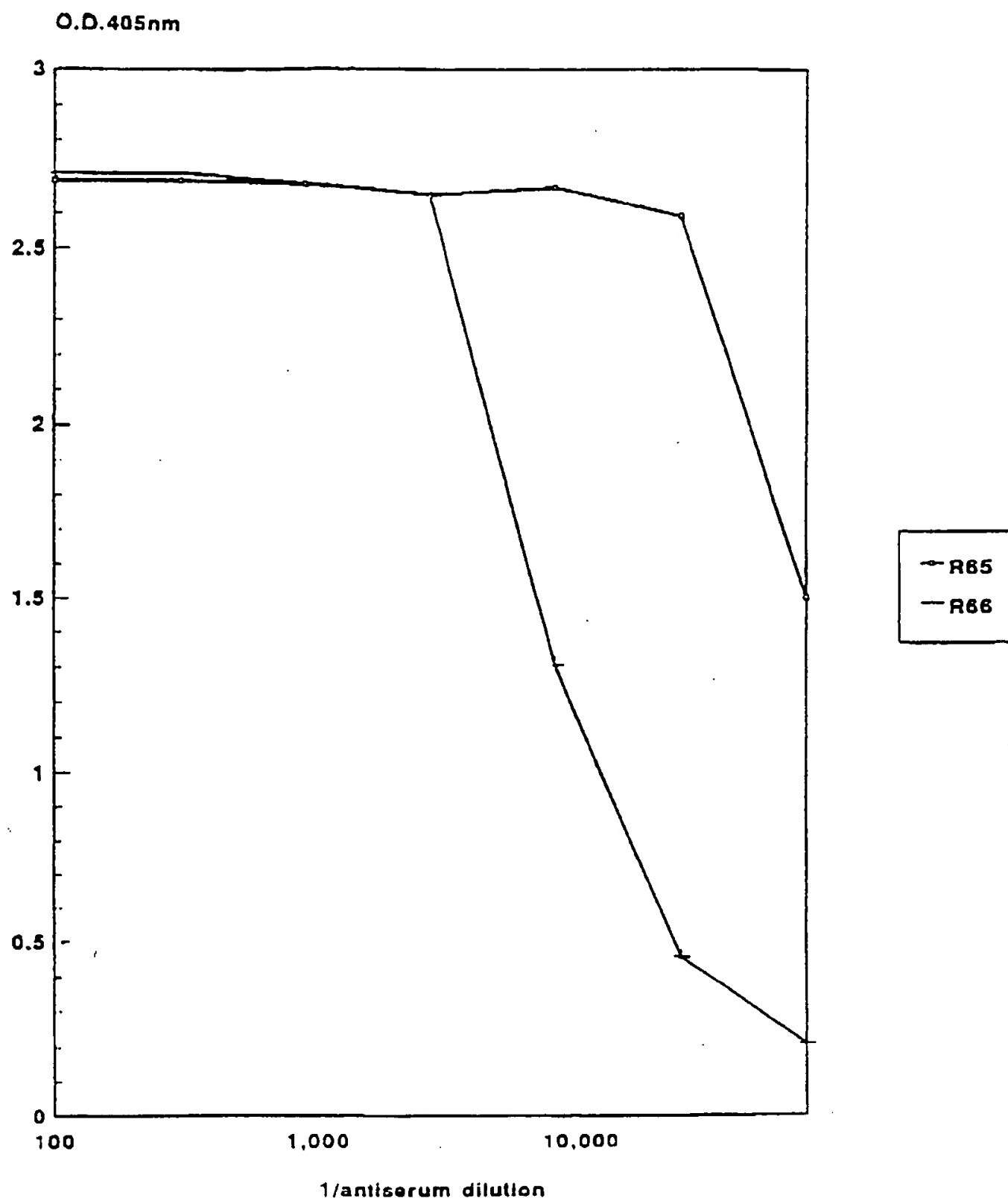
**Figure 2B**  
Inhibition of radiolabelled PDGF-BB binding by PDGF-derived peptides  
Human aortic smooth muscle cells, passage 5



Radiolabelled PDGF at 10ng/ml.  
P7: unrelated peptide.

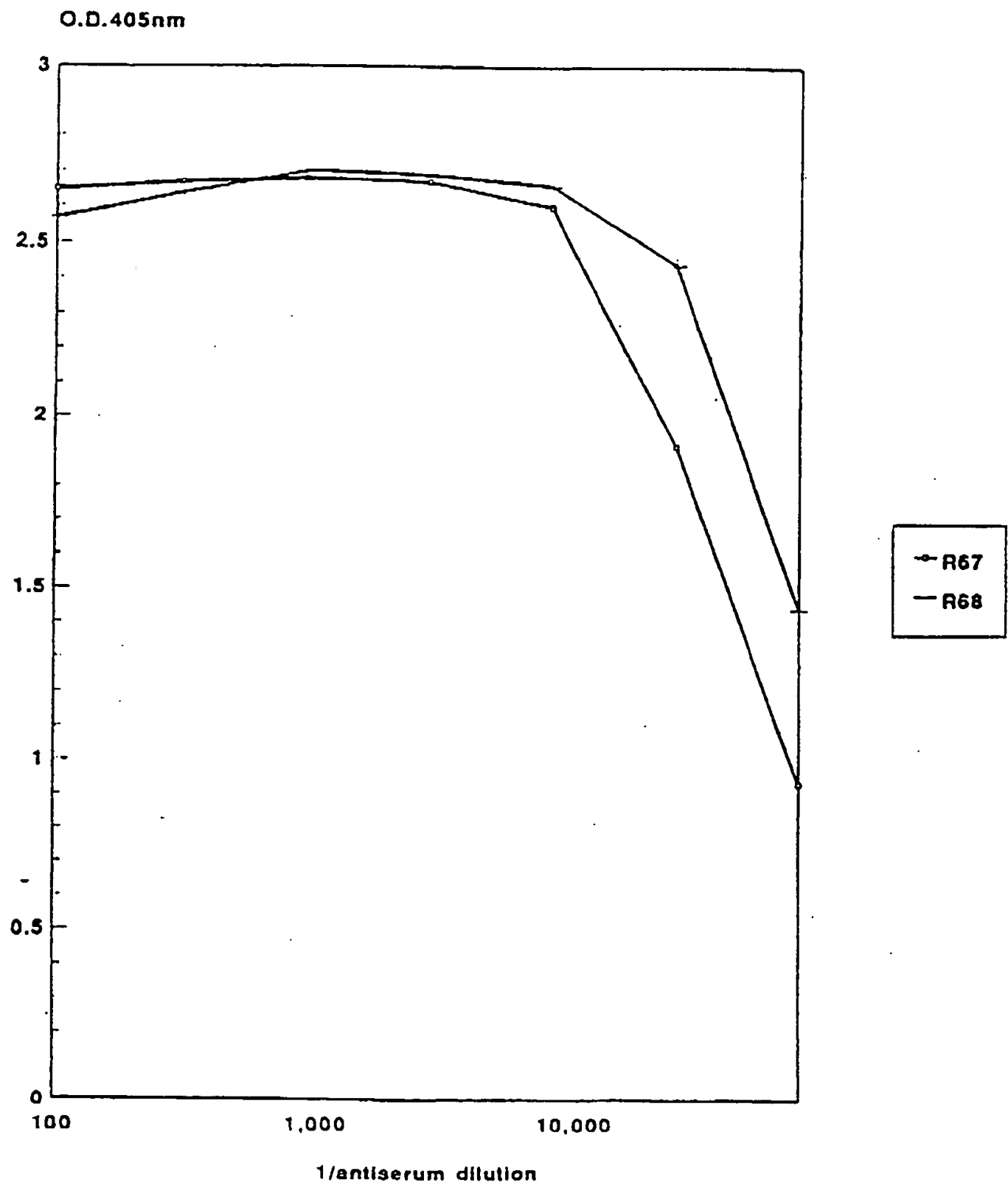
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**Figure 3A**  
Titration of anti-Tg-GP4 vs. GP4(2ug/ml)



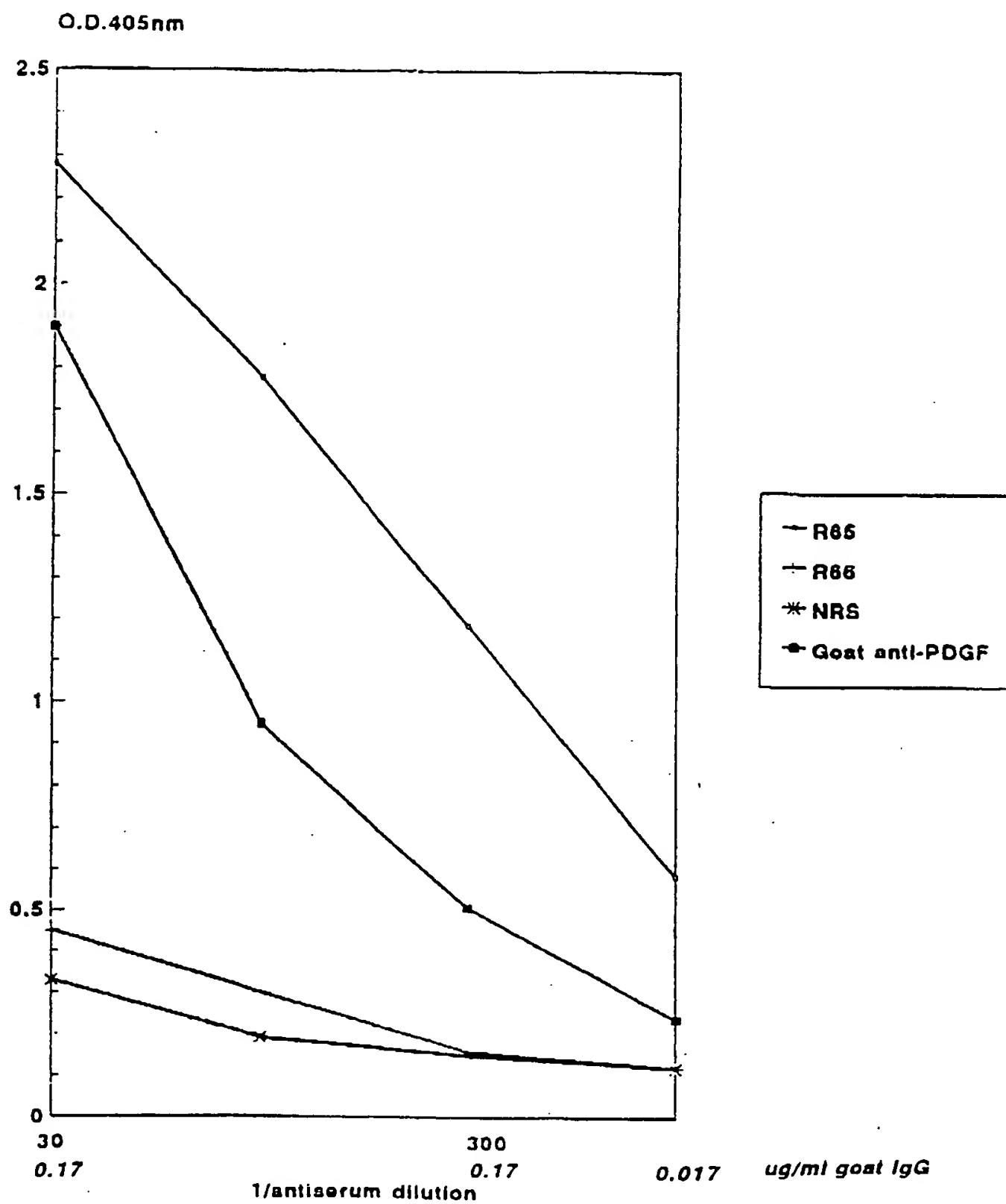
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**Figure 3B**  
Titration of anti-Tg-GP8 vs. GP8 (2ug/ml)



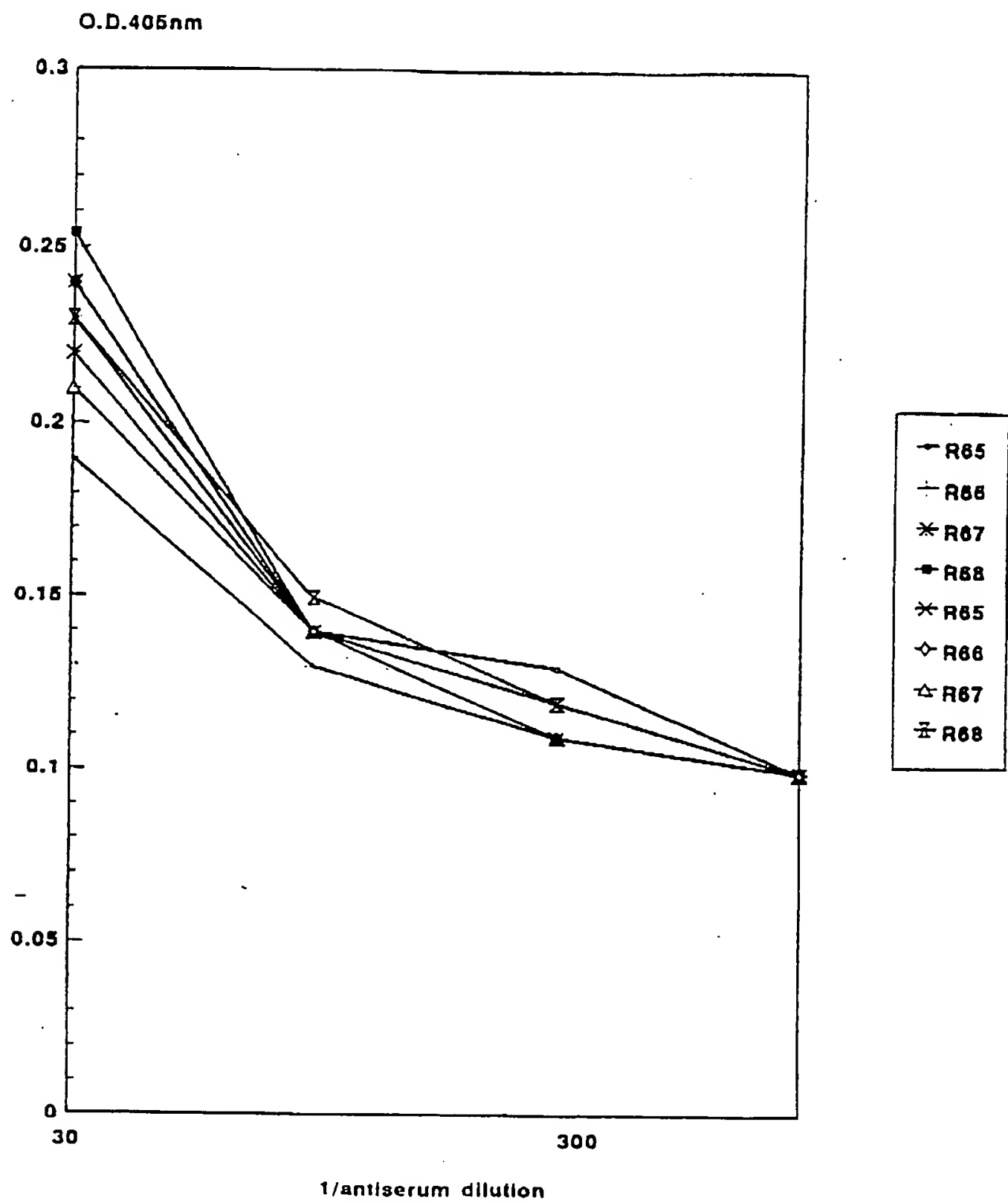
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**Figure 4A**  
Titration of anti-Tg-GP4 vs. PDGF-BB(200ng/ml)

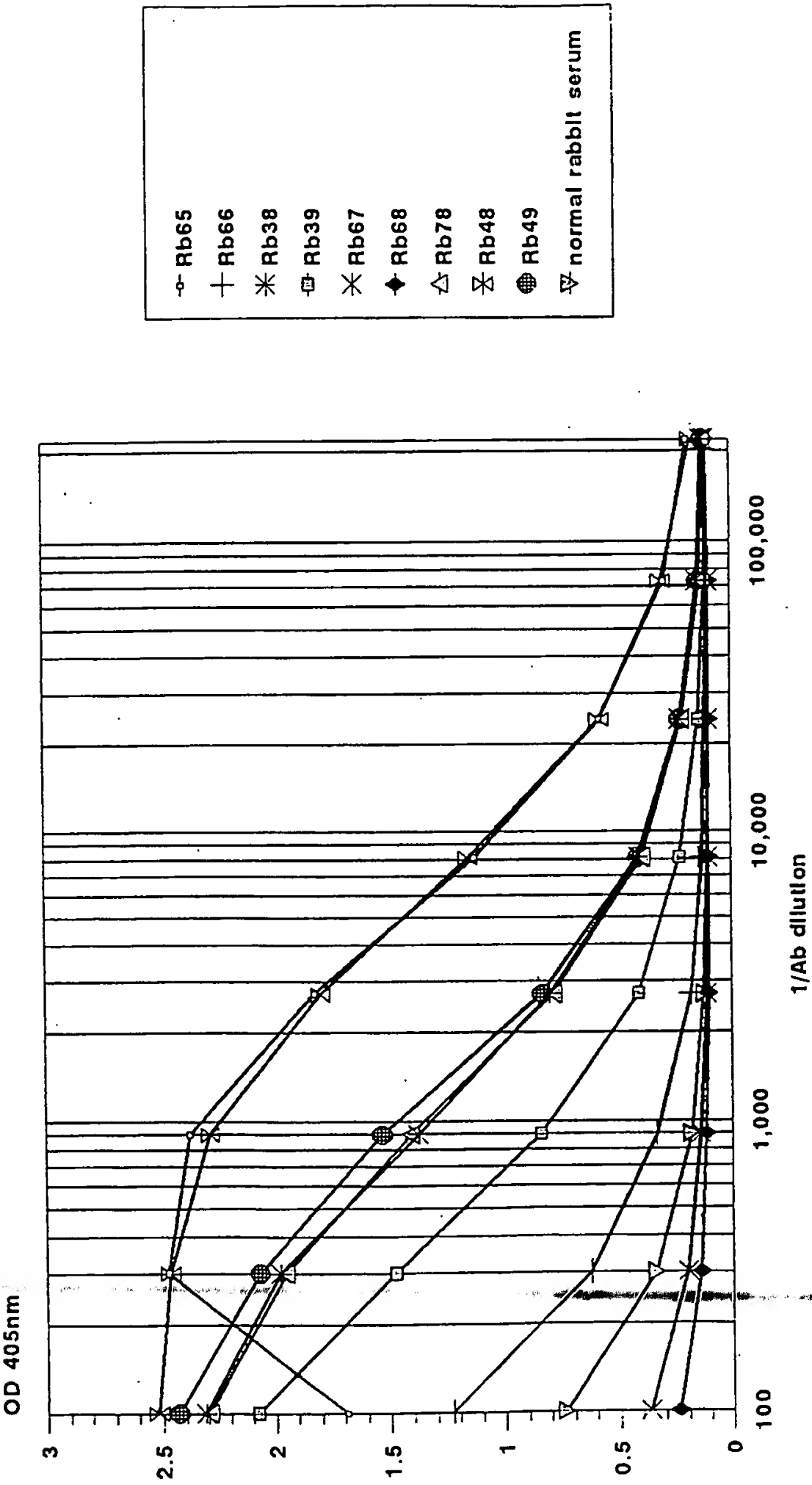


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**Figure 4B**

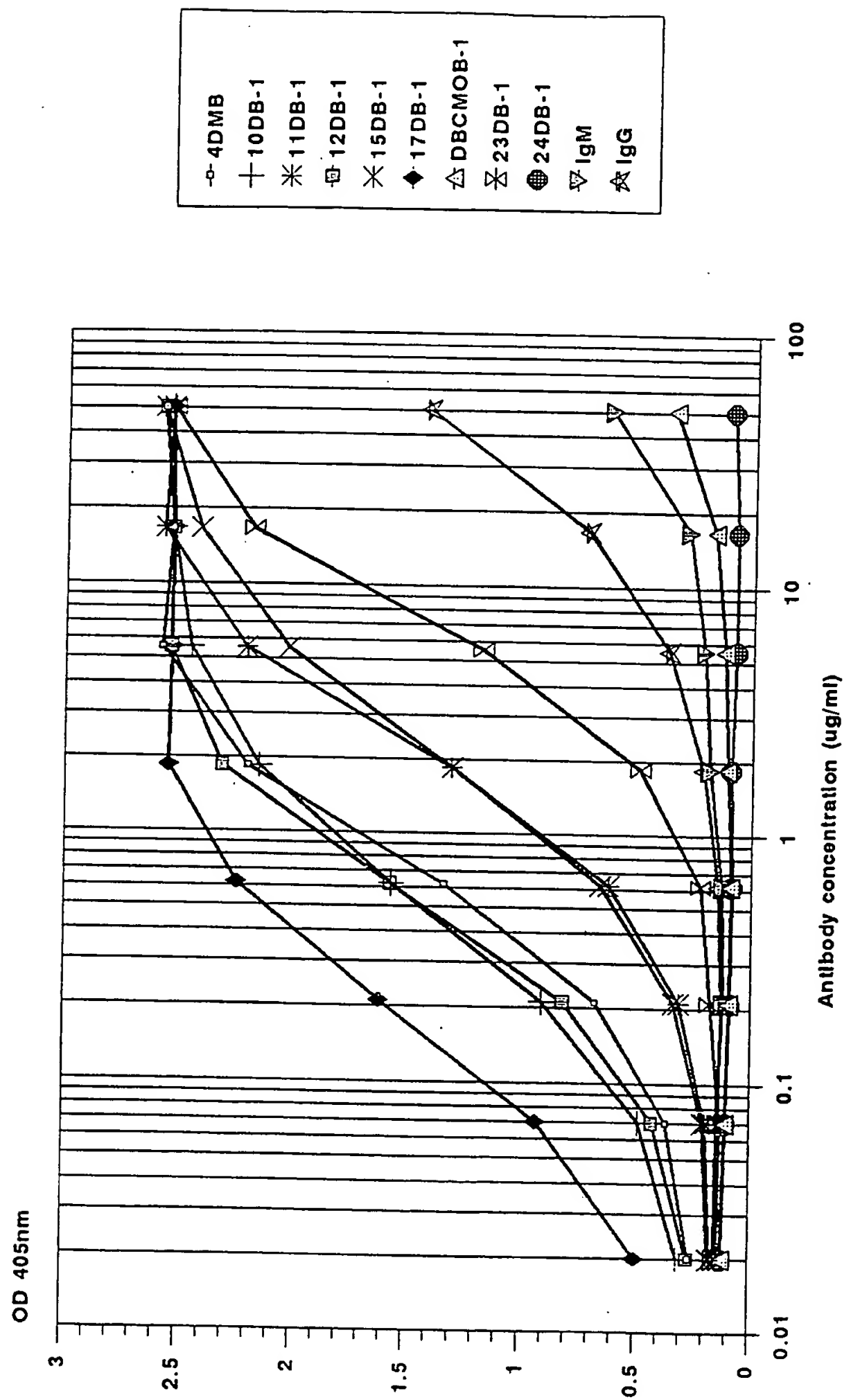


**Figure 5A**  
Direct ELISA Polyclonal Antibody titration. Coating: PDGF-BB, 500ng/ml



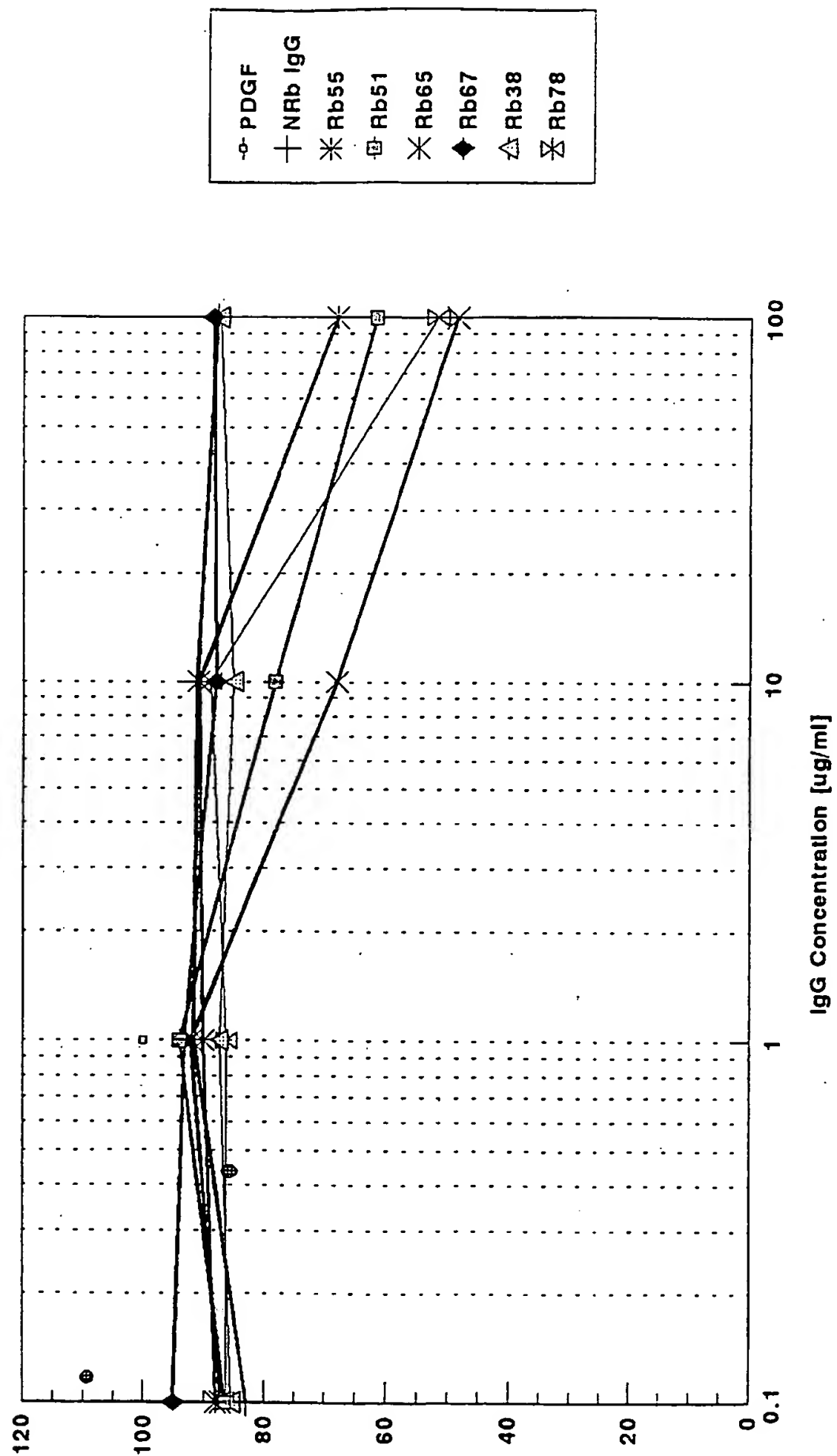
Background signal is 0.109

**Figure 5B**  
Direct ELISA using purified monoclonal antibodies versus PDGF, 500ng/ml



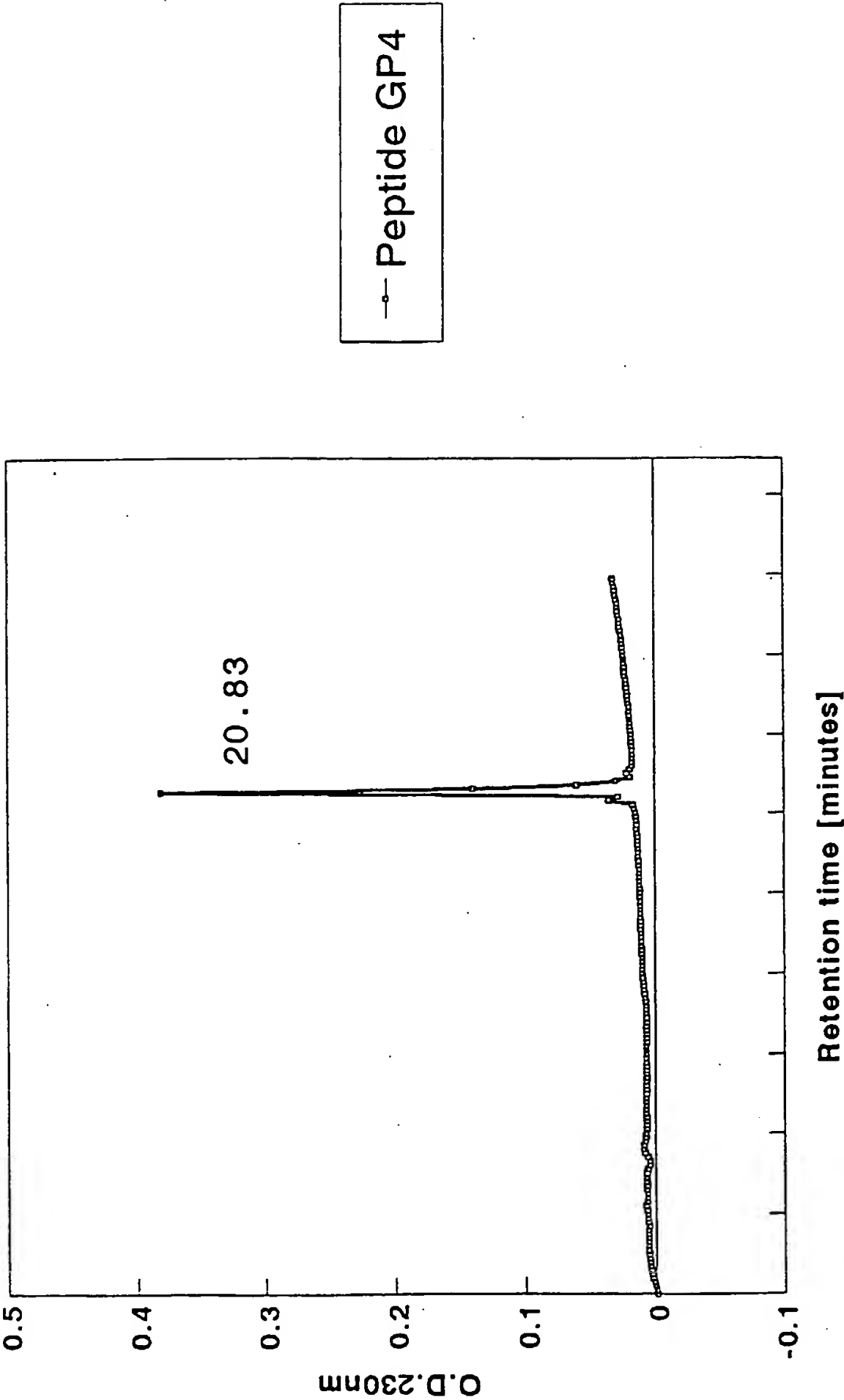
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**Figure 6**  
Anti-PDGF peptides antibodies inhibition of radiolabelled PDGF  
Human aortic smooth muscle cells, passage 5



96-well Falcon plates, 4000cells/well.

**Figure 7A**  
HPLC:C18 Analytical reverse-phase column  
Peptide GP4

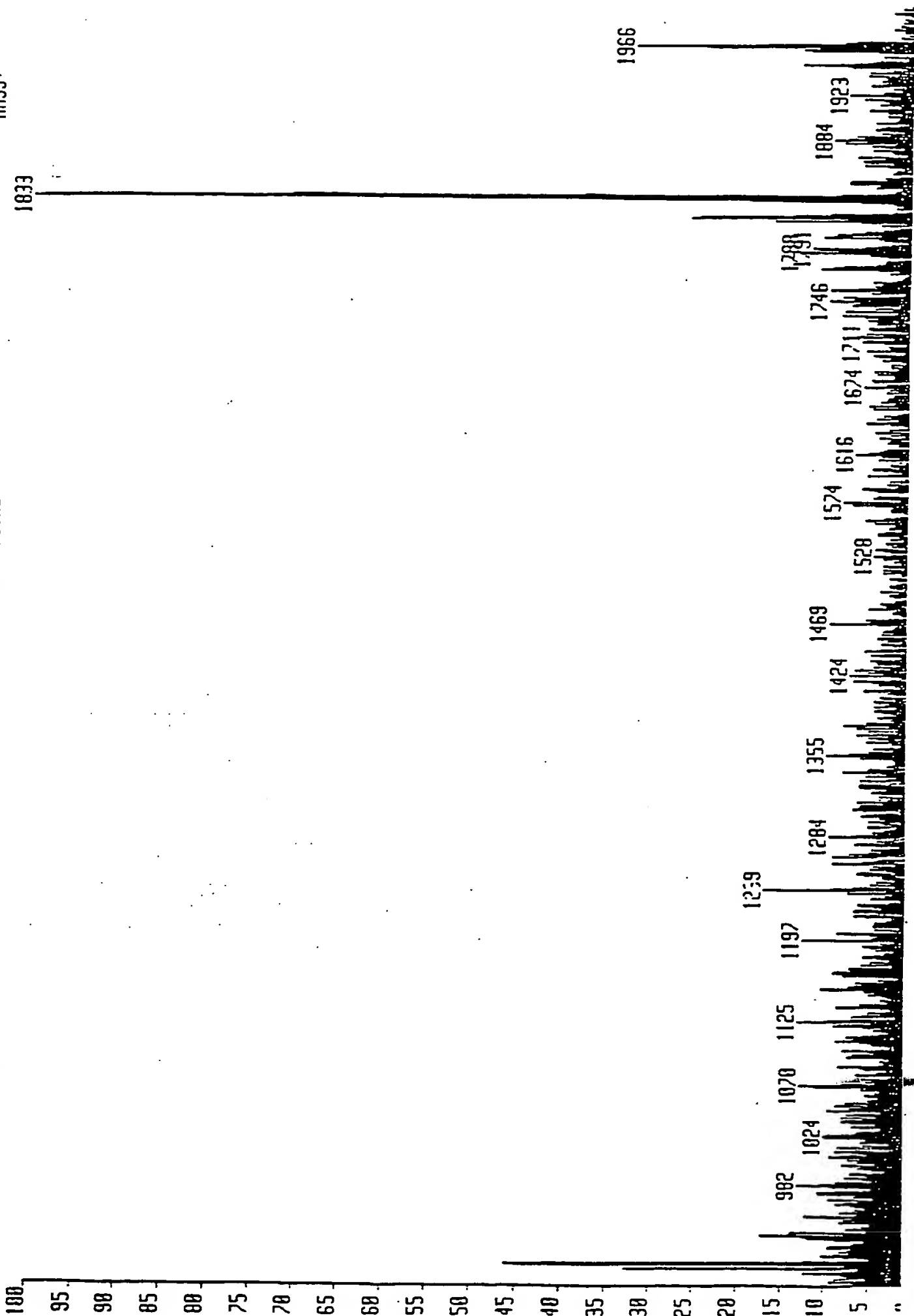


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Figure 7B

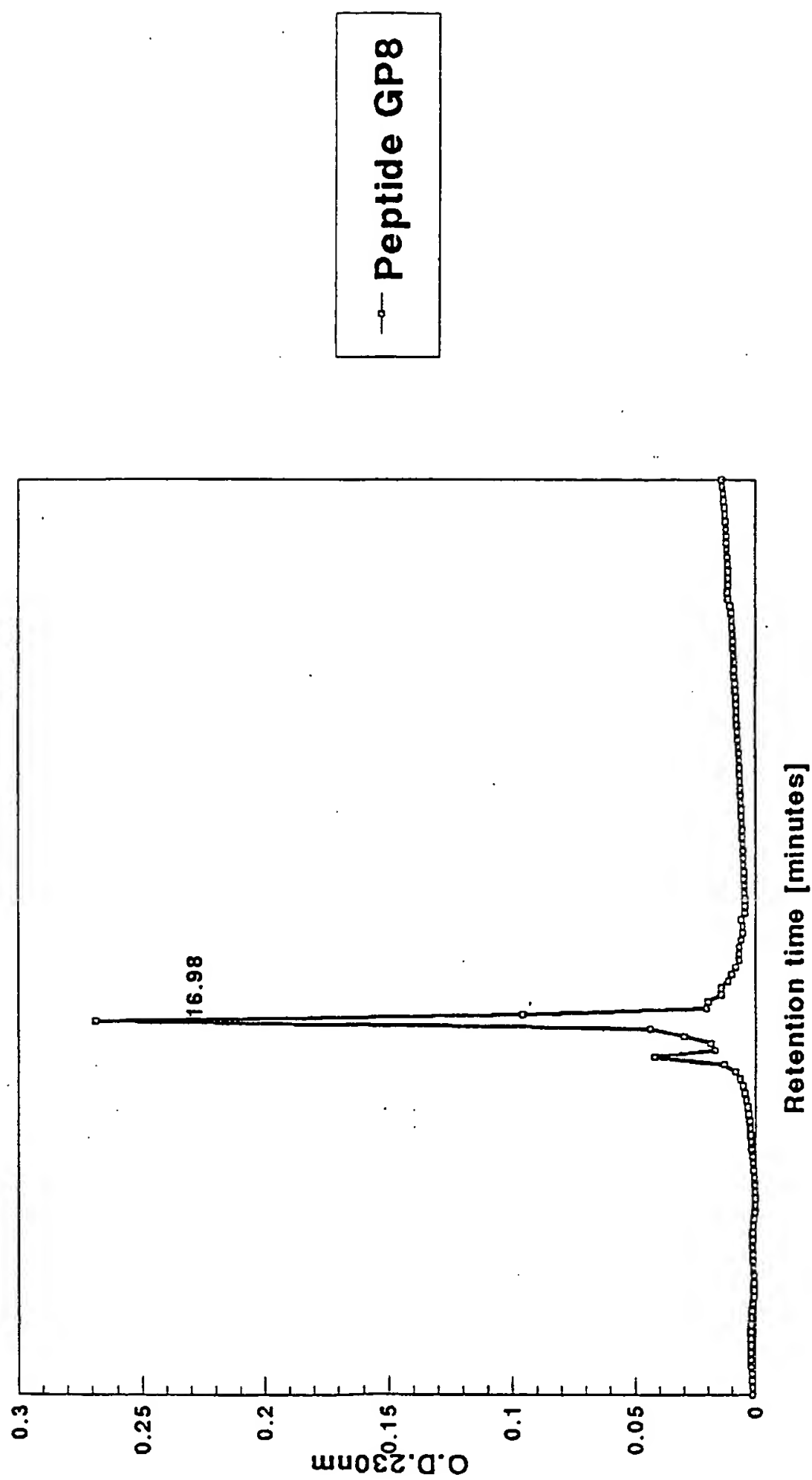
GP4 AC-15RRU1DRTNANFLC  
(Loop 1)

93SE31001 x10 Bgd=0 09-MAR-93 00:30:00:00 ZAB-SE FB+  
8pM=0 I=2.2v Hn=900 TIC=106577296 PV Acnt:ULSOP Sys:HMLRTAB  
GP-14 FAB+ MATRIX: THIOGLYCEROL + GLYCEROL + TFA PI= 0° Cal:FABCAL



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**Figure 8A**  
HPLC : C18 analytical reverse-phase column  
Peptide GP8

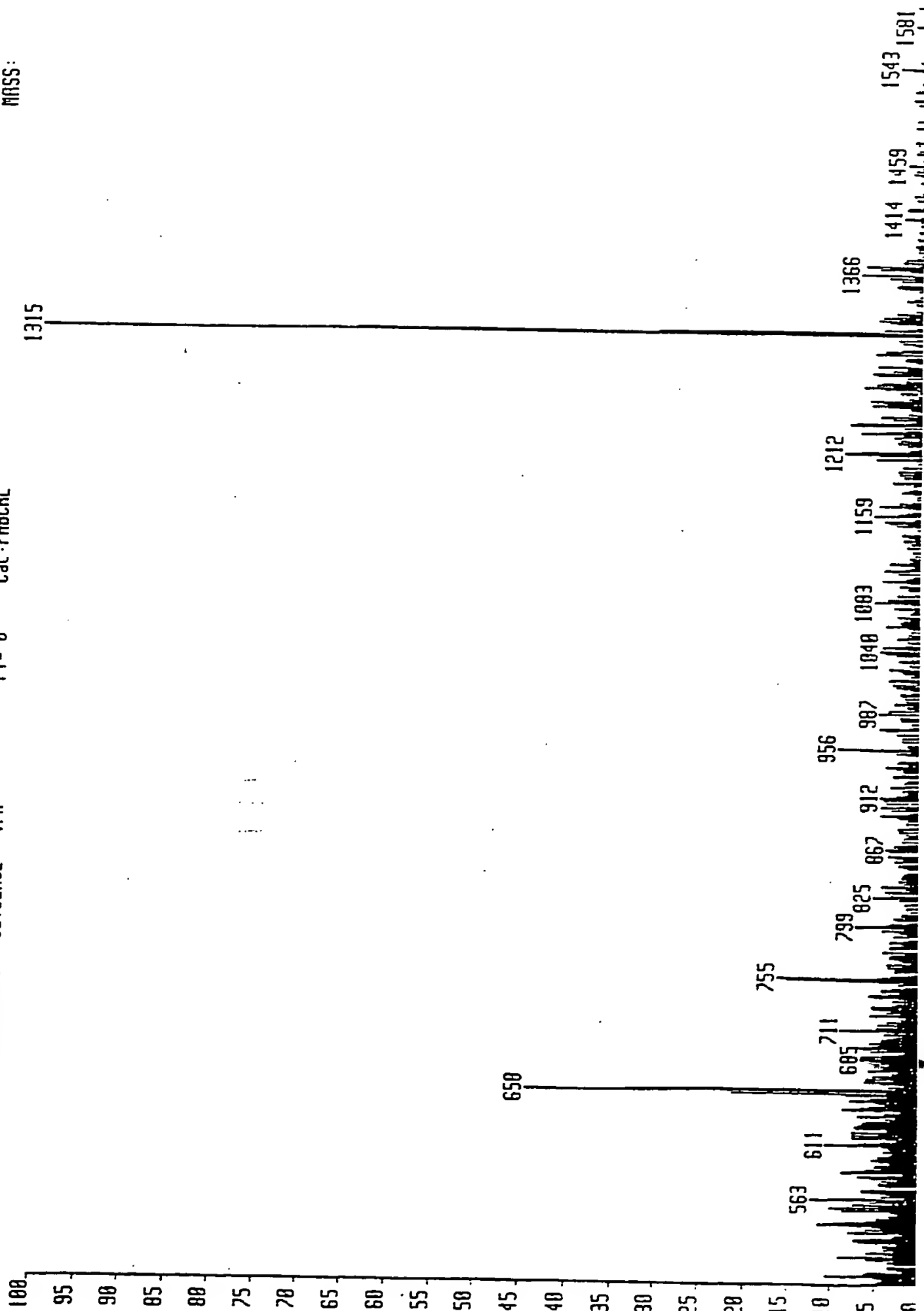


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Figure 8B

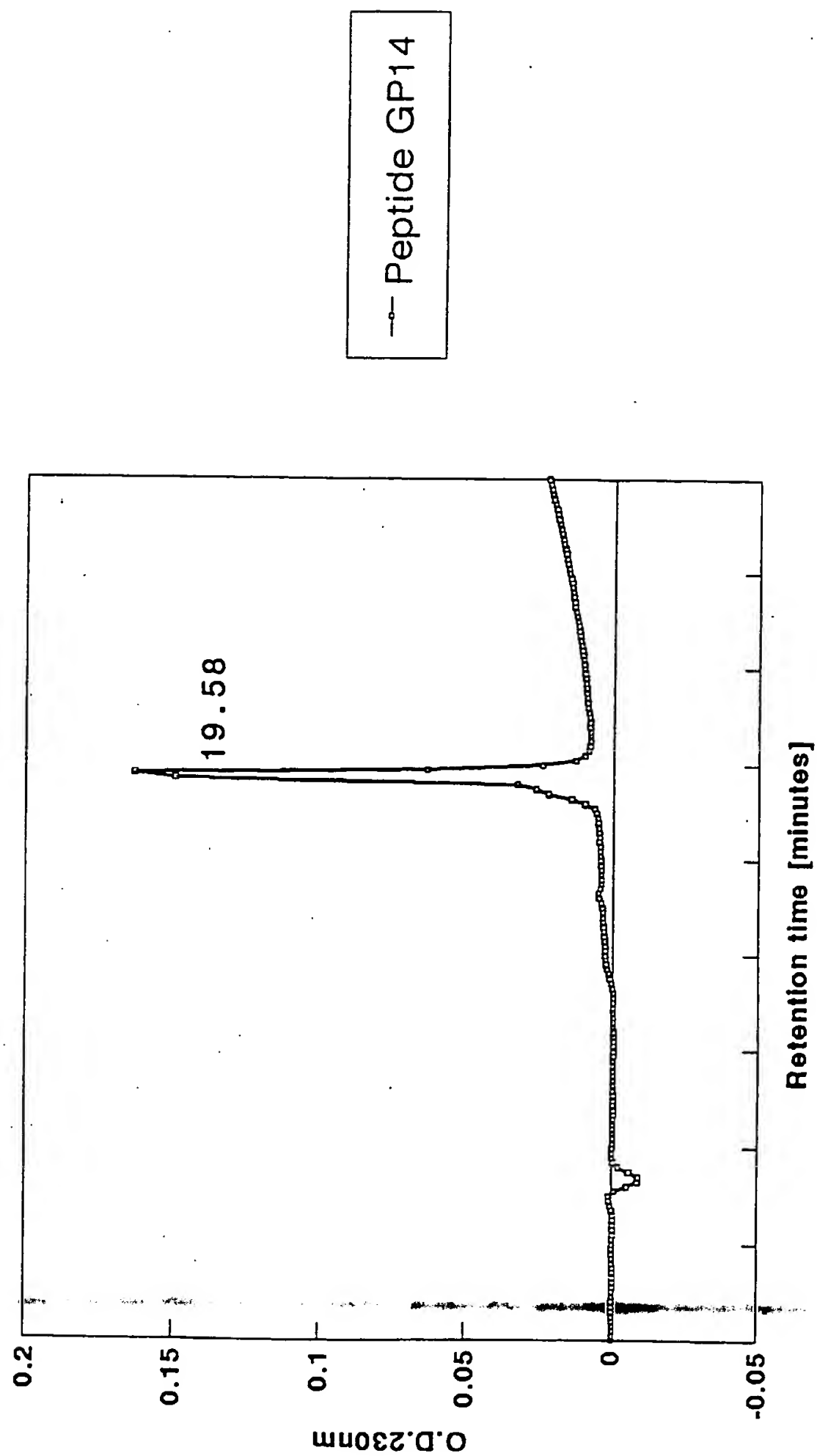
GP8 Ac-RKIEIVRKKC (Loop III)

93SE30911 x1 Bgd=0 05-MAR-93 14:10:00:00 ZAB-SE FB+  
 BpM=0 I=2.4v Hm=900 TIC=166595000 AV Sys: LMLRTAB  
 GP-15 FAB+ MATRIX: THIOGLYCEROL + GLYCEROL + TFA Cal: FABCAL  
 HMR: 1512000  
 MASS: 1315

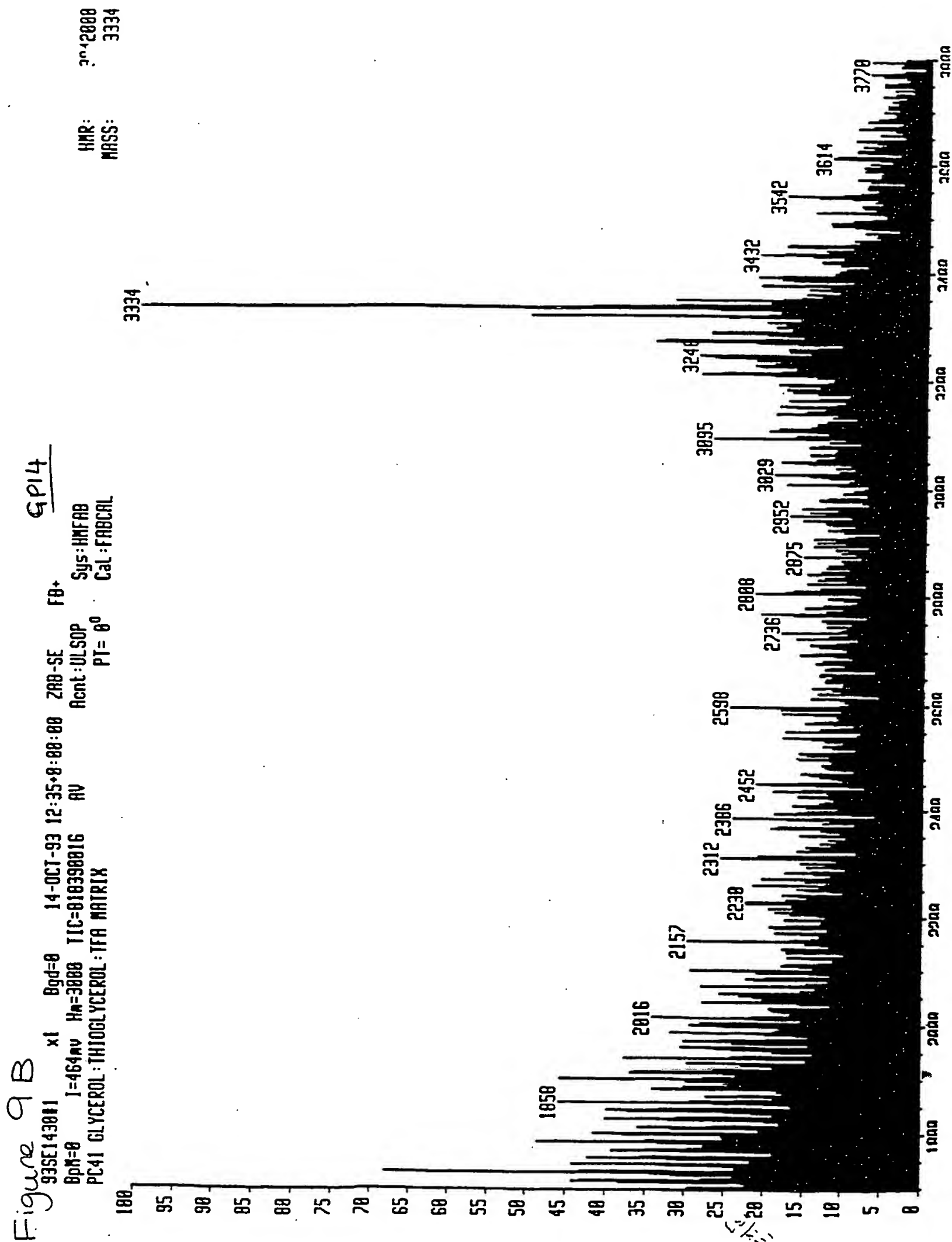


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**Figure 9A**  
HPLC:C18 Analytical reverse-phase column  
Peptide GP14



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PCT/GB 94/02331

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C07K14/49 A61K38/18

According to International Patent Classification (IPC) or to both national classification and IPC

# B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

# C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 16719 (ALLERGAN INC) 2 September 1993  see the whole document ---	2,3, 10-12, 15,17, 19-25
A	JOURNAL OF BIOLOGICAL CHEMISTRY., vol.268, no.14, 15 May 1993, BALTIMORE US pages 10482 - 10489 R FENSTERMAKER ET AL. 'A cationic region of the platelet-derived growth factor (PDGF) A-chain (Arg159-Lys160-Lys161) is required for receptor binding and mitogenic activity of the PDGF-AA homodimer' cited in the application see abstract and introduction --- -/--	1-25

☒ Further documents are listed in the continuation of ...

☒ Patent family members are listed in annex.

## \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
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- \*&\* document member of the same patent family

Date of the actual completion of the international search

9 February 1995

Date of mailing of the international search report

23. 02. 95

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Authorized officer

Masturzo, P

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF BIOLOGICAL CHEMISTRY., vol.267, no.23, 15 August 1992, BALTIMORE US pages 16581 - 16587 U ENGSTRÖM ET AL. 'Identification of a peptide antagonist for platelet-derived growth factor' cited in the application see figure 1 ---	1-25
P,X	WO,A,93 25576 (SRI INTERNATIONAL INC.) 23 December 1993 see the whole document ---	1-25
P,X	WO,A,93 23068 (LUDWIG INSTITUTE FOR CANCER RESEARCH) 25 November 1993 see table 3 -----	1-25

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 15-23 refer to a method of treatment of the human body, the search was carried out and based on the alleged effects of the products.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9316719	02-09-93	AU-B- 3779393 CA-A- 2130748	13-09-93 02-09-93
WO-A-9325576	23-12-93	NONE	
WO-A-9323068	25-11-93	US-A- 5326695 AU-B- 4248293 CA-A- 2135749	05-07-94 13-12-93 25-11-93